

PDK REGULATED WARBURG EFFECT PROTECTS DIFFERENTIATED  
ADIPOCYTES AGAINST ROS

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## **Dedication**

I dedicate this thesis dissertation to my family. To my wife, your patience and support has been a lifeline through these busy years, and I can't imagine having traveled this road without you. To my two boys, it has been a joy to see you grow; I can't wait to see all that you will become. To my parents, your guidance has been paramount throughout my life; undoubtedly, I would not be here today were it not for you.

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ROS

Literature has demonstrated the ability of human adipose tissue to generate large amounts of lactate. However, it is not understood why adipose tissue produces lactate, how the production of lactate is regulated, and what potential benefit this has to the adipocyte or the organism. We first characterized a human model of adipogenic differentiation with minimal donor to donor variability to assess metabolic changes associated with mature adipocytes compared to their precursors. Indeed, similar to what was observed in human clinical studies, the differentiated adipocytes demonstrated increased lactate production. However, the differentiated adipocytes compared to their precursors (preadipocytes or ASCs) demonstrate an aerobic glycolysis-like (also called Warburg effect-like) increase in glycolysis characterized by a 5.2 fold increase in lactate production in normoxic conditions (atmospheric oxygen tension). Remarkably, this increase in lactate occurred even though the differentiated adipocytes simultaneously demonstrate an increase in oxidative capacity. This low fraction of oxidative capacity coupled with increased lactate production indicated regulation of oxidative rates most likely at the point of pyruvate conversion to either acetyl-CoA (oxidative metabolism) or lactate (glycolytic metabolism). To investigate the potential regulation of this metabolic phenotype, PDK isoform expression was assessed

and we found PDK 1 and 4 transcript and protein elevated in the differentiated cells.

Non-selective pharmacologic inhibition of the PDKs resulted in decreased lactate production, supporting a regulatory role for PDK in modulation of the observed Warburg effect. PDK inhibition also resulted in increased ROS production in the adipocytes after several hours of treatment and a decrease in cell viability when PDK inhibition was carried out over 36 hours. The resulting loss in viability could be rescued by antioxidant (Tempol) treatment, indicating the decrease in viability was ROS mediated. Similar to what is seen in cancer cells, our data demonstrate that differentiation of human adipocytes is accompanied by a PDK-dependent increase in glycolytic metabolism (Warburg effect) that not only leads to lactate production, but also seems to protect the cells from increased and detrimental generation of ROS.

Keith L March, MD, Ph.D., Chair

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## Abbreviations

1,3BPG	1,3-bisphosphoglycerate
2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
acetyl-CoA	acetyl-Coenzyme A
ADP	adenosine diphosphate
ARIC	Athero Risk in Communities
ATP	adenosine triphosphate
CHOP	C/EBP homologous protein
CoQ	coenzyme Q
DHAP	dihydroxyacetone phosphate
DNA	deoxyribonucleic acid
ETC	electron transport chain
F1,6BP	fructose 1,6-bisphosphate
F6P	fructose 6-phosphate
FADH <sub>2</sub>	flavin adenine dinucleotide (hydroquinone form)

FMN	flavin mononucleotide
FOXO1	forkhead box protein O1
G6P	glucose 6-phosphate
GADP	glyceraldehyde 3-phosphate
GAPDH	glyceraldehyde phosphate dehydrogenase
GTP	guanosine triphosphate
HIF-1 $\alpha$	hypoxia inducible factor-1 alpha
LDH	lactate dehydrogenase
mTORC1	mammalian target of rapamycin complex 1
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide plus hydrogen
NEFA	non-esterified fatty acid
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PDC	pyruvate dehydrogenase complex
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase



PEP	phosphoenolpyruvate
PGK	phosphoglycerate kinase
Pi	Phosphate
QH2	Ubiquinol
RET	reverse electron transport
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
succinyl-CoA	succinyl-Coenzyme A
TCA	tricarboxylic acid
TNF $\alpha$	tumor necrosis factor alpha
UPR	unfolded protein response

## **Chapter I:**

### **Introduction**

#### **A. Obesity**

##### **A.1 Introduction**

Obesity, defined generally as an excess in adipose tissue, has rapidly become one of the most prevalent healthcare issues facing the world. There are a number of comorbidities affecting quality and duration of life associated with obesity. Its prevalence has expanded to the point where it has achieved epidemic levels and has become one of the leading causes of healthcare expense in the United States.

##### **A.2 Pathophysiologies associated with obesity**

There is much literature demonstrating the association of cardiovascular disease and type 2 diabetes with obesity. These are summed up very well in several comprehensive reviews that demonstrate obesity (and pathogenic fat or adiposopathy often associated with hypertrophied adipocytes) is a significant comorbidity with CVD, type 2 diabetes, hypertension, and kidney disease [1-11]. Further demonstrating the association of excess adiposity with these comorbidities, weight loss demonstrates a positive effect in prevention of certain aspects of metabolic syndrome and obesity related comorbidities and can even reverse some of the effects [12, 13].

In addition to the comorbidities often associated with obesity and the metabolic syndrome listed above, a number of other pathophysiologies are associated with obesity such as sleep apnea [14-19] and gastroesophageal reflux disease (GERD) [20, 21]. Non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) are more prevalent in obese populations and directly correlate with increased adiposity in individuals [22-26]. A number of cancers demonstrate high degrees of correlation with obesity including breast cancer [27-29], endometrial cancer [30-32], colorectal cancer [33-37], renal cell carcinoma [38-40], ovarian cancer [41-43], and prostate cancer [44-49]. Adiposity also contributes to musculoskeletal pathologies including joint pain [50-52] and osteoarthritis (OA) [51, 53-58].

### **A.3 Costs associated with obesity**

Obesity correlates with a reduction in life expectancy largely due to the comorbidities previously mentioned. A body mass index (BMI) greater than or equal to 30 is considered obese. BMIs of 30-35 and 40-50 reduce life span by 3 years and 10 years respectively [59]. Such effects on lifespan are similar to the effects of smoking. Often not explored however are the costs associated with obesity though the total cost for obesity related medical expenditures is high [60]. A near linear correlation exists between increase in healthcare costs and BMI that increases from 20-40 [61]. Medical resources allocated per obese individual is estimated to be one third higher than individuals with healthier BMIs [62].

#### **A.4 Measuring adipose tissue mass and obesity**

Considering the important physiologic role of adipose tissue and pathophysiologic impact of excess adiposity in humans, many methods have been developed for assessing adipose tissue mass and classify obesity. Obesity is most often clinically defined by BMI. BMI is an attempt to estimate (more accurately qualify) adipose tissue mass in humans using indirect measures. It is calculated as weight (pounds) multiplied by 703 divided by height (inches) squared, or as weight (kilograms) divided by height (meters) squared. Severe thinness is considered a BMI of less than 16, moderate thinness is 16-16.9, mild thinness is 17-18.49, normal is 18.5-24.9, overweight is 25-29.9 and obese is greater than or equal to 30. There are three to four (depending on reference) subcategories of obesity: class I is BMI 30-34.9, class II is 35-39.9, and class III is greater than or equal to 40 [63]

While BMI is widely used, alternative measurements for classification of obesity and their ability to correlate with obesity comorbidities can be used. These are valuable since BMI can be misleading as an indirect measure of obesity that does not take into account fat vs lean mass. For example, BMI does not reflect the increase in fat mass and decrease in muscle mass that occurs in the elderly. As a result, portly elderly persons with low muscle mass often have underestimated body fat with low reported BMIs [64]. Conversely, individuals with high muscle mass such as professional athletes tend to have overestimation of obesity. For example, in the National Football League, one in four athletes are considered extremely obese ( $\text{BMI} \geq 35$ ) [65], however, their

mean body fat percentage when directly measured is 14%, well within very healthy ranges [66]. The relationship between actual body fat percentage and BMI is non-linear [67] and the fact that much of the data has been accrued using BMI as an index for obesity has caused improper classifications [68].

Other measurements exist as alternatives to BMI that often demonstrate increased correlation with the incidence of obesity comorbidities. Abdominal indicators have been identified as potentially more accurate discriminators of cardiovascular risk [69]. Waist circumference demonstrates positive correlation for predicting Type II Diabetes in obese populations [70]; waist to height ratio has a better correlation than BMI for the obesity related comorbidities hypertension, lipidemia, and Type II Diabetes [71]; waist to hip ratio has demonstrated better correlation than BMI for cardiovascular risk factors associated with obesity in some studies [72]. Beyond abdominal and other anthropomorphic indices, other measurements such as impedance has been suggested as a more accurate classification tool for adiposity [73]. Nevertheless, BMI is still most commonly used since it is easily obtained and large amounts of data have already been accrued using this method.

#### **A.5 Obesity prevalence**

Regardless of method for assessing obesity, it affects an alarming number of individuals. Using the NHANES data, trends in the frequency of obesity have been tracked by collecting height and weight data for which BMI is calculated. Data from 1988-1994 showed an increase in obesity of 8 percent over data from 1977-1980 with

prior data collection from 1960-1980 levels of obesity remaining level [74, 75]. Looking further, data from 1999-2000 demonstrated increased obesity as well [76]. Data from 2001-2002 and 2003-2004 demonstrated increasing trends among men but not women compared to 1999-2000 data [77, 78]. The 2003-2004 data from the 2005-2006 and 2007-2008 data showed no real increase in prevalence of obesity [21].

While there has not been a dramatic increase in obesity within the past several years, the overweight and obese population in the United States is alarmingly high. Specifically, from the most recent NHANES data (2009-2010) on overweight and obesity prevalence in the US, the prevalence of obesity adjusted for age was 35.7%. For adult men prevalence was 35.5% overall. For women, prevalence was 35.8%. No significant difference existed among age adjusted prevalence of obesity between men and women ( $P = 0.86$ ). Combining both overweight ( $BMI \geq 25$ ) and obese ( $BMI \geq 30$ ), age adjusted prevalence among adults was 68.8% with 73.9% of men and 63.7% of women meeting that criteria. Numbers are similarly high among various ethnicities within the United States. Among men and women tested, 34.3% of non-Hispanic white, 49.5% of non-Hispanic black, 39.1% of Hispanic, and 40.4% of Mexican Americans were obese. Overweight and obese populations combined again demonstrated alarmingly high rates: 66.7% of non-Hispanic white, 76.7% of non-Hispanic black, 78.8% of Hispanic, and 81.2% of Mexican American individuals demonstrated this phenotype. All of this data was obtained from summaries by Flegal et al [79].

Perhaps even more alarming is the high degree of obesity among children and adolescents in the US. Clinical trends identify obese children at higher risk for development of adult obesity and morbid obesity placing obese youth at risk for the same comorbidities later life [80, 81]. This trend in obesity leads to increased risk factors in adults. One study demonstrates being overweight during adolescence associates with an increase of 2.4-3 fold in high total cholesterol and LDL, an 8.5-fold increase in hypertension, and an 8-fold increase in reduced high-density lipoprotein cholesterol in adults[82]. Another study found that increasing BMI during the age range of 7-13 correlates with increasing prevalence of coronary events later in life [83]. Current childhood obesity trends from 1999-2010 are well characterized by Ogen et al [84]. Briefly, in youth through age 19, obesity prevalence was 16.9% and overweight prevalence was 31.8%. Of these data, males demonstrated a higher percentage of obesity compared to females, 18.6 vs. 15.0% respectively ( $P = 0.01$ ). While childhood obesity growth rates have somewhat plateaued [85]; the overall prevalence of obesity across all ages is alarmingly elevated.

#### **A.6 Obesity summary**

With multiple comorbidities affecting mortality and quality of life, the prevalence of and costs associated with obesity have become a major health concern worldwide and especially in the United States. This has increased the need for research into obesity. Physiologically, these research studies focus on adipose tissue and more specifically on adipocyte biology, its normal and pathophysiologic function. By studying

this particular cell type, adipose tissue function is better defined which leads to a better understanding of the biology at the center of the obesity epidemic. This dissertation falls into this important research category and provides a better understanding of human adipocyte biology with respect to its differentiation and metabolic phenotype.

## **B. Adipose Tissue**

### **B.1 Introduction**

In the previous section, a number of ways have been introduced that are used to categorize and quantify obesity, however all of them attempt to estimate lean vs. fat mass. Fat mass is almost entirely adipose tissue. Some extra adipose tissues/organs such as brain, marrow and liver/skeletal muscle (when fatty) can contribute to more sensitive quantitative methods (such as QMR), however, contribution of extra-adipose lipid to whole animal or human fat/lean measurements is negligible compared to adipose tissue itself. This said, clinical obesity is qualitatively described as an increased body mass particularly in adipose tissue sufficiently large enough to produce adverse health consequences[86]. This increase in adipose tissue is primarily due adipogenic differentiation of adipocyte precursors as well as hypertrophy of. These processes are driven by energy imbalance (net positive caloric balance) and hormonal regulation but also can be influenced by genetic components.



## B.2 Genetic component of adipose tissue formation and obesity

Through clinical studies in humans where genetic abnormalities contribute to changes in adipose tissue mass, genetic components that contribute to regulation of adipose tissue mass have been identified. This is challenging however, since a series of molecular mechanisms have been cloned and defined that regulate energy homeostasis [87], even though in animal studies, body size is polygenic with no Mendelian pattern as it is controlled by multiple loci. In humans, identifying a genetic component of obesity is even more daunting as environmental variables contribute to feeding behavior and energy expenditure. However, evidence exists that variance of BMI within a population can be genetically determined [87, 88] with some estimates of heritability ranging between 40-70% [89] though such studies are difficult to account for similar environmental factors affecting parents/offspring. Association, linkage, and candidate gene studies have identified a number of human obesity associated genes. These include *LEP* (leptin deficiency) [90-92], *LEPR* (leptin receptor deficiency) [93, 94], *POMC* (proopiomelanocortin deficiency) [95, 96], *PCSK1* (proprotein convertase 1/3 deficiency) [97, 98], *MC4R* (melanocortin 4 receptor deficiency) [99-102], *BDNF* (brain derived neurotrophic factor deficiency) [103, 104], *NTRK2* (neurotrophic tyrosine receptor kinase 2 deficiency) [105], *SIM1* (single minded homolog 1 deficiency) [106], *BBS1-16*, (Bardet Biedl Syndrome) [107], *ALMS1* (Alstrom Syndrome) [108], *GNAS* (Albright Hereditary Osteodystrophy) [109], with new genes still being identified. While these studies indicate a clear genetic component to obesity, most are rare genetic abnormalities and do not largely contribute to population obesity in a major way.

### **B.3 Energy imbalance and formation of adipose tissue**

As informative as the genetic association studies have been, the increased prevalence of obesity over the last several decades especially in the United States is most likely not due to a sudden change in Western World genetics. It is more likely due to change in lifestyle and availability/composition of food consumed. Whether genetic, environmental, and/or behavioral, an increase in fat mass is essentially a shift in energy balance. Assuming that physiologic nutrient absorption and storage of energy is functioning within normal parameters, stored energy only increases when energy intake exceeds energy expenditure. In this positive caloric state, excess energy storage is most optimally stored in new adipocytes created by adipogenic differentiation of existing adipocyte precursors (discussed in more detail later) [86, 110-113]. Hypertrophy of existing adipocytes is also involved in storage of excess energy in positive caloric balance. Initially, when in a state of excess energy, hypertrophy of existing adipocytes triggers adipose tissue paracrine signaling resulting in differentiation of new adipocytes [114-116].

### **B.4 Adipocyte number and hypertrophy**

Historically, a prevailing hypothesis was that human have a fixed number of adipocytes determined early on and kept constant regardless of environmental/physiologic conditions throughout life. Thus an increase in adipose tissue mass was thought to be entirely due to hypertrophy of existing adipocytes and that this this fixed number of adipocytes (in excess or insufficiency) might predispose to

obesity and its comorbidities [117]. This has been shown to not be the case. Increase in fat mass also occurs by recruitment/proliferation of preadipocytes which undergo adipogenic differentiation increasing energy storage capacity in adipose tissue [114, 118-122]. Thus, expansion of fat mass is a balance of hypertrophy of existing adipocytes and differentiation of new adipocytes. It is important to note however that when hypertrophy exceeds normal physiologic parameters (resulting from insufficient new adipocyte differentiation leading to increased adipocyte size) a pathologic state often exists in adipose tissue that drives many of the pathophysiologies previously described associated with obesity [1-4, 110, 123-132]. Differentiation of new adipocytes, thereby expanding the capacity of adipose tissue to store energy without over-hypertrophy of existing adipocytes, is critical for normal adipose tissue function.

### **B.5 Adipose tissue as an endocrine organ**

While classically described as an energy storage depot, additionally adipose tissue has more recently been characterized as having an endocrine role with extensive hormone production [133-135]. As early as 1987 sex steroid metabolism [136] and adiponectin production [137] were described in adipose tissue. Since then, a number of adipokines have been identified with endocrine and paracrine action regulating multiple in vivo systems. These include but are not limited to... leptin [138-140], adiponectin [141-145], resistin [146-150], chemerin [151-153], omentin [154], retinol binding protein 4 (RBP4) [155-157], TNF $\alpha$  [138, 139, 158, 159], IL-6 [138, 139, 160, 161], MCP-1 [162-164], plasminogen activator inhibitor (PAI)-1 [165], and multiple components of the

rennin angiotensin system (RAS) [166, 167]. Adipokine production and regulation is critical for normal physiologic function both systemically and within adipose tissue, regulating multiple functions contributing to several pathophysiologies when dysregulated [86, 110, 113, 168-170].

## **B.6 Adipocyte differentiation**

During adipocyte differentiation, preadipocytes with no lipid droplets differentiate into mature adipocytes filled with perlipin coated lipid vesicles. This involves several stages. Adipose tissue contains mesenchymal precursors with proliferative capacity and plasticity to differentiate. Under adipogenic conditions, these cells then go on to become committed preadipocytes that still have the ability to proliferate. These further go on to growth arrest and differentiation into mature adipocytes[112, 171].

## **B.7 Intracellular regulation of adipocyte differentiation**

A number of transcription factors have been identified that regulate adipogenic differentiation, with two emerging as the most critical for this process: PPAR $\gamma$  and C/EBP $\alpha$ . Peroxisome proliferator-activated receptor gamma, or PPAR $\gamma$ , is considered to be the master regulator of adipocyte differentiation. PPAR $\gamma$  forms a heterodimer with RXR to bind DNA. The target of this heterodimer is the DR-1 sequence, characterized by repeats of 'AGGTCA' with a random nucleotide between them [172]. PPAR $\gamma$  is critical for adipogenic induction and sufficient to induce adipogenesis [173, 174]. Both PPAR $\gamma$ 1 and 2 have been shown to contribute to adipocyte differentiation [175-178]. PPAR

gamma ligand is upregulated by C/EBP $\beta$  and SREBP1C [179, 180]. Specific endogenous PPAR $\gamma$  ligands remain elusive though lipid metabolites such as polyunsaturated fatty acids and eicosanoids have been implicated as potential candidates [181]; Prostaglandin 15-deoxy- $\Delta$ 12, and 14-prostaglandin J2 have been considered potential endogenous ligands although their expression is low in adipocytes [182, 183]. cAMP-dependent ligand activity is temporarily elevated early in differentiation though PPAR $\gamma$  expression remains elevated throughout differentiation [184] indicating that continued receptor agonism is not necessary for PPAR $\gamma$  expression. Further, inducible PPAR $\gamma$  knockout *in vivo* [185] and introduction of a dominant negative PPAR $\gamma$  *in vitro* [186] have demonstrated that continued expression of PPAR $\gamma$  is critical for adipogenic differentiation and mature adipocyte function.

Also critical for adipose tissue differentiation is CCAAT-enhancer-binding-protein alpha (C/EBP $\alpha$ ). This directly induces many adipocyte genes and is itself induced by C/EBP $\beta$  and C/EBP $\delta$  [187-189]. Other members of the C/EBP family suppress adipogenesis potentially through heterodimerization and inactivation of C/EBP $\beta$  [187]. Though important for differentiation, C/EBP can not function apart from PPAR $\gamma$  [190, 191]. C/EBPs continue to play a role in mature adipocytes to maintain insulin sensitivity [192, 193].

While PPAR $\gamma$  and C/EBP $\alpha$  are considered the most critical regulators of adipogenesis, a number of other intracellular factors have been identified as regulating different aspects of adipogenic differentiation both downstream of PPAR $\gamma$  and C/EBP $\alpha$

and/or contributing to their elevation. These include but are not limited to KLF2, 5, 7, 15 [194-198], LXRs ( $\alpha$  and  $\beta$ ) [199-201], STAT5a [202, 203], CREB [204], Krox-20 [205], early beta cell factor (EBF) [206], and SREBP1 [180, 207, 208].

## **B.8 Extracellular signal regulation of adipocyte differentiation**

Wnts are a family of highly conserved secreted glycoproteins that influence cell fate and development by either paracrine and/or autocrine action with affecting multiple organs and tissues such as bone, intestine, hematopoiesis of multiple white blood cells[209]. Wnt signaling occurs through both  $\beta$ -catenin driven pathways (canonical) and other non-canonical pathways [209, 210] that regulate adipogenic differentiation. Wnt signaling inhibits *in vitro* adipogenic differentiation by blocking PPAR $\gamma$  and C/EBP $\alpha$  expression [200, 211-213]. Selective deletion studies during embryogenesis suggest that Wnt proteins serve a similar regulatory role of adipogenic differentiation *in vivo* [214]. Along with Wnts, a number of other extracellular signaling proteins have been identified as playing a role in regulating adipogenic differentiation. The transforming growth factor  $\beta$  (TGF $\beta$ ) family members have demonstrated various regulatory roles on adipogenesis; these include TGF $\beta$  [215-220], myostatin [221-226], and bone morphogenic proteins (BMPs) [227-232]. Notch signaling has also been implicated in adipocyte differentiation and is regulated by a number of factors including jagged and  $\gamma$ -secretase [233-238]. DLK1/PREF1 signaling represses adipogenic differentiation [239-244]. Fibroblast growth factors (FGFs) are generally pro-adipogenic signaling [245-249]. These factors have been identified in controlled experiments

attempting to identify the role of individual factors. The net regulation of adipogenesis *in vivo* is a concert of all these factors and others as is the case with most differentiation events.

### **B.9 Modeling adipogenesis *in vitro***

Most of what has been learned about adipocyte differentiation has come from *in vitro* models of adipocyte differentiation. A number of cell-based models exist, the most commonly used are 3T3-L1 and 3T3-F442A cells. These have been used in an impressive body of literature that has greatly aided in our understanding of adipocyte differentiation and adipocyte function. These clonal lines of fibroblasts were initially isolated from Swiss 3T3 mouse embryos [250-252].

While these embryonic mouse fibroblast lines have contributed to the understanding of adipogenesis, human models using human adipocyte precursors may be preferable to more appropriately model human adipogenic differentiation. This can be done using the stromal vascular fraction of adipose tissue, containing adipose-derived stromal cells (ASCs), which can be readily obtained through minimally invasive lipoaspiration as well as abdominoplasty. This adipose stromal cell population is largely composed of adipocyte precursors and the MSCs that give rise to adipocyte precursors [253]. While the stromal vascular population out of processed lipoaspirate contains ASCs it also contains a large number of endothelial and leukocytic cells [254]. Thus, this population represents the cells that contribute to normal adipogenic differentiation *in vivo*. It seems reasonable that study of adipogenic differentiation of these cells is using

the “right cell from the right tissue, in the right species,” and provides a more physiologic model for studying human adipogenic differentiation than the previously mentioned 3T3 lines. ASCs have demonstrated multilineage potential including *in vitro* differentiation into mesenchymal cells [255-258] as well as hepatocytes [259], neuronal cells [260], endothelial cells [261, 262], and cardiomyocytes [263, 264]. ASCs occupy a perivascular niche and support vascular networks [254, 265, 266] in addition to contributing to new adipocyte formation.

Adipocyte differentiation is achieved by multiple (similar) methods depending on the source of cells for differentiation and the extent of differentiation desired. Differentiation is most commonly induced by treating confluent cells with a cocktail of insulin, dexamethasone, and isobutylmethylxanthine (IBMX). This cocktail was originally defined for 3T3-L1 cells and was then translated into use for human adipocyte precursor differentiation [267-269]. For cell types in a more undifferentiated state (not committed preadipocytes), commitment and progression through differentiation can be enhanced with addition of PPAR $\gamma$  agonists such as Pioglitazone and Rosiglitazone to the differentiation medium. This is important for the stromal vascular populations of adipose tissue which contain mesenchymal stem cells and preadipocytes at various stages of adipogenic commitment. For cells already committed, PPAR $\gamma$  agonists increase PPAR $\gamma$  expression. Thus PPAR $\gamma$  agonists enhance adipogenic differentiation in cell lines and primary isolated human ASCs [270-274]. However, direct comparison of the effects of PPAR $\gamma$  agonism on human preadipocytes from different depots is not very well defined and is explored further in this dissertation.



## **B.10 Adipose tissue summary**

Human ASCs provide a species and tissue appropriate physiologic model for studying adipogenic differentiation and even a method for providing mature adipocytes for research in a more controlled manner than fat pad culture. However, the degree of donor-to-donor variability in differentiation needs to be assessed for human adipocyte studies. While differentiation capacity of omental derived ASCs is reduced compared to subcutaneous with respect to lipid accumulation, percentage of cells differentiating, AP2, PPAR $\gamma$ , and C/EBP $\alpha$  expression [275], enhancing commitment and differentiation procedure with PPAR $\gamma$  agonists might improve differentiation studies in omental depots.

Differentiation from a pericytic niche and role to a role that is in large part energy storage most likely is associated with a drastic change in metabolic phenotype. This dissertation research aims primarily at exploring the changes in metabolic phenotype that accompany adipogenic differentiation.

## **C. Lactate Production in Adipose Tissue**

### **C.1 Introduction**

Lactate production primarily comes from the conversion of glycolytically-derived pyruvate to lactate by lactate dehydrogenase under hypoxic conditions. This was initially thought to primarily occur in skeletal muscle during periods of intense exercise

where oxygen delivery may not be sufficient for energy utilization. However, adipose tissue has also demonstrated lactate production in a regulated manner.

## **C.2 Physiologic lactate production**

Lactate had typically been thought to be produced under circumstances where conditions are not favorable for oxidative phosphorylation. Briefly, glucose is transported into cells and phosphorylated to prevent passive export. It is then catabolized by a series of enzymes into two three carbon molecules of pyruvate. This pyruvate is then either transported into the mitochondria to be oxidized or it is converted to lactate via lactic acid fermentation. Lactate dehydrogenase catalyzes the conversion of pyruvate to lactate (and the reverse as well). Essentially the pyruvate from glycolysis is reduced to lactate by NADH (which is oxidized to  $\text{NAD}^+$ ). When conditions are not permissive for oxidation (hypoxia), this step is critical to replenish the  $\text{NAD}^+$  necessary for the initial glycolytic breakdown of glucose entering the cell. Lactate can passively leave the cell which can enter the blood stream for uptake at the liver where it can either be used as substrate for glucose production (gluconeogenesis) or it can be taken back up into the cells that produced it and converted to glycogen (primarily liver and skeletal muscle) . The production of glucose or storage as glycogen from systemic lactate in the liver is known as the Cori cycle [276-278]. This is considered a futile cycle as it requires additional energy to “reuse” the carbons in lactate to generate glucose or store as glycogen.

Lactate production has classically thought to occur in tissues of non-obligate anaerobes when anaerobic metabolism is necessary due to a lack of sufficient oxygen to catabolize glucose by oxidative phosphorylation. This is discussed further in the next section but is exemplified by skeletal muscle during periods of intense contraction in which oxygen is not sufficient to provide ATP through oxidative phosphorylation [279-281]. Cancer cells have also demonstrated production of ATP through an enhanced glycolytic phenotype resulting in increased lactate production [282-285] which will be reviewed in detail in the next section.

### **C.3 Lactate production from adipose tissue**

Studies performed nearly two decades ago demonstrated a significant contribution to systemic lactate by adipose tissue [286]. Systemic lactate levels change independent of exercise and are readily altered by feeding [287, 288] and lactate levels rise after a meal with a nearly 20 minute lag time in relation to the rise in glucose and insulin [289, 290]. Skeletal muscle and splanchnic bed, the common sources of lactate production, do not contribute to this observed increase in systemic lactate after feeding [289, 291-293] suggesting a non-skeletal muscle contribution to blood lactate. Up to the early 1980s, lactate production from adipose tissue was mentioned but not heavily studied [294-298]. Then in the 1980s and 1990s, glucose metabolism to lactate by adipose tissue and focused studies on adipocytes began to demonstrate that adipose tissue is a significant source of systemic lactate that varies with experimental conditions and is potentially associated with pathophysiologies [299-304].

A number of factors have been experimentally associated with changes in lactate production by adipocytes. Increased adipocyte size in rats correlates with increased conversion of glucose to lactate [301-305] which is similar to findings in humans that increased lactate production is observed in adipocytes from obese individuals [299, 300, 306]. Insulin [303] and epinephrine [305] both increase lactate production in isolated rat adipocytes. Adipocytes isolated from rats fed a high fat chow also demonstrated elevated glucose conversion to lactate as compared to rats fed normal chow [294]. Each of these demonstrate that adipose tissue is not only capable of contributing to systemic lactate, but that such processes are regulated.

Perhaps the most significant characterization of adipose lactate production analysis was achieved by human studies of adipose tissue in vivo by direct adipose tissue microdialysis and abdominal wall venous drainage studies. A small number of human studies identified elevated concentrations of lactate in the adipose tissue interstitial space compared to circulating plasma levels during fasting, and an even greater elevation after glucose load [307, 308]. Similar findings were found when comparing venous and arterialized plasma from subcutaneous adipose tissue in the abdominal wall separate from the underlying muscle [309, 310]. Microdialysis studies in humans also demonstrated elevated lactate production in obese vs. lean individuals [306, 311]. Similar to rodent blood lactate/adipocyte size correlation, circulating lactate increases in correlation with adipocyte size [312] and in obese versus lean humans [290, 313-315], and normoglycemic first-degree relatives of individuals with type 2 diabetes whose

adipocytes have demonstrated increased size as well [316, 317]. Taken together, these studies clearly demonstrate adipose tissue as a producer of systemic lactate.

As far as two decades ago a positive correlation between elevated fasting lactate and a reduction in insulin sensitivity has been reported [286, 290, 318]. However, acute increase in plasma lactate production following meal, glucose challenge, or euglycemic clamp study is decreased with a progressive increase in insulin resistance [319-322]. In the Atherosclerosis Risk in Communities (ARIC) study examining a cohort of over 8000 predominantly black and white adults ages 45-64 over two decades, it was found that baseline plasma lactate (adjusted for other risk factors) demonstrates positive association with diabetes, fasting glucose, and insulin [323]. The underlying mechanism suspected to be driving insulin resistance involves gluconeogenic response to elevated lactate in the liver resulting in chronic hyperglycemia [286, 324]. While this has been held up in rodent clamp/lactate infusion studies [325, 326], acute studies in humans have not demonstrated similar elevation of glucose [291, 327-329] though the effects of long term plasma lactate elevation have not been studied requiring further and more rigorous experimentation.

#### **C.4 Lactate production in adipose tissue summary**

It is clear from literature that lactate is produced in adipose tissue in a regulated manner and correlates with alterations in insulin sensitivity. However, why it is produced in adipose tissue is poorly understood. Further discussed in the next section, lactate production as a result of glucose metabolism is energy inefficient. Also, how this

production is regulated in adipocytes and the consequences of inhibiting its production in these cells is not well-understood. This dissertation explores this at a cellular level using a model of human adipocyte differentiation.

## **D. Aerobic Glycolysis (Warburg Effect)**

### **D.1 Introduction**

Cells generally metabolize glucose to pyruvate through glycolysis. Under normoxic conditions, pyruvate is further metabolized to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  generating NADH to power electron transport and ATP generation through oxidative phosphorylation. When oxygen is not present or limited, pyruvate is converted to lactate through lactic acid fermentation. However, in some unique cases, lactate generation occurs in normoxic conditions, this is generally called aerobic glycolysis or the Warburg effect.

### **D.2 Glycolysis/pyruvate production**

Glycolysis is generally divided into two phases, the preparatory phase which generates two three-carbon sugar phosphates (glyceraldehydes 3-phosphate) while consuming energy (2 ATP) followed by the the payoff phase which generates energy-rich ATP and NADH. Each reaction of the payoff phase occurs twice per glucose molecule (end of preparatory phase generates two substrate for the payoff phase). The net energy yield of glycolysis is 2 NADH and 2 ATP molecules (4 ATP are generated in the payoff phase, but 2 are consumed in the preparatory phase).

In more detail, the first step of the preparatory phase involves the phosphorylation of glucose by hexokinases forming glucose 6-phosphate (G6P). G6P is then rearranged (isomerized) by glucose phosphate isomerase to form fructose 6-phosphate (F6P). F6P is phosphorylated by phosphofructokinase 1 to generate fructose 1,6-bisphosphate (F1,6BP). F1,6BP is split by aldolase into two molecules forming dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GADP). DHAP is then interconverted by triosephosphate isomerase to form a second GADP.

The two GADPs then move into the payoff phase, with each of the following reactions occurring twice. GADP is dehydrogenated and inorganic phosphate is added forming 1,3-bisphosphoglycerate (1,3BPG) by glyceraldehyde phosphate dehydrogenase (GAPDH) which also results in the generation of NADH from  $\text{NAD}^+$ . The 1,3BPG contributes one phosphate to ADP forming ATP and 3-phosphoglycerate (3PG) by phosphoglycerate kinase (PGK); this substrate level phosphorylation producing ATP (one of two steps generating in glycolysis). 3PG is then converted to 2-phosphoglycerate (2PG) by phosphoglycerate mutase. 2PG then becomes phosphoenolpyruvate (PEP) through the action of enolase. Finally PEP forms pyruvate transferring one more phosphate to ADP through the action of pyruvate kinase (the second substrate level phosphorylation). This results in the formation of pyruvate and an additional ATP completing the payoff phase and glycolytic catabolism of glucose.

Glycolysis can ultimately be written as the following:  $\text{glucose} + 2 \text{NAD}^+ + 2 \text{ADP} + 2 \text{P}_i \rightarrow 2 \text{pyruvate} + 2 \text{NADH} + 2 \text{H}^+ + 2 \text{ATP} + 2 \text{H}_2\text{O}$ . If this process were to continue for

ATP generation with no additional reactions, glycolysis would eventually stop with all the cellular  $\text{NAD}^+$  in the cell being used and not replenished. For glycolysis to continue, cells must oxidize NADH back to  $\text{NAD}^+$ . Generally, under conditions where oxygen is not present, pyruvate undergoes a process known as lactic acid fermentation, generating lactate and  $\text{NAD}^+$ . When performing oxidative metabolism,  $\text{NAD}^+$  for glycolysis is generally replenished by two methods.

Under normoxic conditions, pyruvate enters into TCA generating even more NADH with oxygen used as the final electron acceptor in the electron transport chain. This generates the needed  $\text{NAD}^+$  and establishes the proton motive force that powers ATP synthase. However, due to their semi-permeable nature, mitochondrial membranes inhibit transport of this  $\text{NAD}^+$  to the cytosol where it is needed to support further glycolysis. To replenish  $\text{NAD}^+$  in the cytosol, cytosolic malate dehydrogenase catalyzes the reaction of NADH with oxaloacetate producing  $\text{NAD}^+$  and malate. The  $\text{NAD}^+$  can be used in glycolysis while the malate is transported into the mitochondria. There, mitochondrial malate dehydrogenase converts it to oxaloacetate, reducing  $\text{NAD}^+$  to form NADH. This oxaloacetate is converted to aspartate by aspartate aminotransferase which produces  $\alpha$ -ketoglutarate from glutamate in the process. The glutamate for this is transported into the mitochondria by an antiporter which simultaneously transports malate into the cytosol. The aspartate is converted back to oxaloacetate in the cytosol by aspartate aminotransferase. This is generally considered the malate-aspartate shuttle. Alternatively,  $\text{NAD}^+$  for glycolysis can be restored through another mechanism known as the glycerol phosphate shuttle. In this process, NADH is



oxidized to  $\text{NAD}^+$  by glycerol-3-phosphate dehydrogenase as it converts dihydroxyacetone phosphate to glycerol 3-phosphate. The  $\text{NAD}^+$  produced can be used to continue glycolysis.

### **D.3 Lactic acid fermentation**

Lactic acid fermentation is considered to be the completion of glycolysis when oxygen is not present, replenishing cellular  $\text{NAD}^+$  for glycolytic metabolism of glucose and subsequent substrate level phosphorylation generation of ATP to occur. This process involves the conversion of pyruvate to lactate and is catalyzed by lactate dehydrogenase (LDH). The overall reaction is as follows:  $\text{pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{lactate} + \text{NAD}^+$ . The use of this process coupled with glycolysis for ATP production is considered glycolytic metabolism.

### **D.4 Oxidative metabolism/TCA cycle**

Under aerobic conditions, pyruvate is converted to acetyl-CoA to enter the citric acid cycle and generates an NADH in the mitochondrial matrix via a process known as pyruvate decarboxylation. This is achieved by action of the pyruvate dehydrogenase complex (PDHC), the regulation of which will be discussed later (see section on PDKs). This critical reaction for pyruvate's fate is sometimes called the link reaction because it links the metabolic pathways of glycolysis with the citric acid cycle.

Acetyl-CoA under aerobic conditions is then fed into the citric acid cycle (also known as the tricarboxylic acid cycle, TCA, or the Krebs cycle) generating carbon dioxide ( $\text{CO}_2$ ) and

NADH from  $\text{NAD}^+$  ultimately used in electron transport. TCA begins with consumption of acetate in the form of acetyl-CoA along with oxaloacetate to form citrate via the action of citrate synthase. The citrate is then converted to cis-Aconitate by aconitase which also catalyzes the next step converting cis-aconitase into isocitrate. This is then turned into  $\alpha$ -ketoglutarate generating NADH by isocitrate dehydrogenase.  $\alpha$ -Ketoglutarate is then turned into succinyl-CoA by  $\alpha$ -ketoglutarate dehydrogenase generating NADH in the process. Succinyl-CoA is then converted to succinate by succinyl-CoA synthetase generating GTP in the process. Succinate is then oxidized to form fumarate and ubiquinol ( $\text{QH}_2$ ) by succinate dehydrogenase. Fumarate is then hydrated to generate malate via fumarase. Malate is in turn converted to oxaloacetate by malate dehydrogenase generating NADH in the process and also providing oxaloacetate to push the initial step of TCA again along with new acetyl-CoA. The overall reaction for TCA can be described as  $\text{acetyl-CoA} + 3\text{NAD}^+ + \text{Q (ubiquinone)} + \text{GDP} + \text{P}_i + 3\text{H}_2\text{O} \rightarrow \text{CoA-SH} + 3\text{NADH} + 3\text{H}^+ + \text{QH}_2 + \text{GTP} + 2\text{CO}_2$ .

#### **D.5 Metabolic yields from glycolytic and oxidative metabolism**

NADH and  $\text{FADH}_2$  (from TCA) are then used as electron donors for the electron transport chain generating the proton motive force across mitochondrial membrane and driving ATP production through ATP synthase. Assuming 3 ATP per NADH and 2 per  $\text{FADH}_2$ , the maximum ATP yield per glucose molecule under aerobic conditions (assuming complete oxidation of glucose) is 38 ATP. However, these processes are not entirely efficient. The two NADH generated in glycolysis cost ATP to transport into the

mitochondria reducing the total ATP yield per glucose to 36. Furthermore, the mitochondrial membrane can “leak” protons reducing the proton motive force and reducing ATP production per NADH/FADH<sub>2</sub>. Taking this into consideration, the net ATP yield per glucose is theoretically approximated closer to 30 ATP.

Comparing the two processes, glycolytic metabolism produces a maximum net 2 ATP per glucose metabolized whereas oxidative metabolism produces a more efficient maximum of 38 ATP per glucose metabolized. Because of this increased efficiency cells generally produce ATP through oxidative metabolism in permissive environments (normoxic conditions). This was first observed in 1857 when Louis Pasteur demonstrated that aerating yeast broth caused yeast cell growth to increase with a decrease in fermentation. **His observation became known as the Pasteur effect which generally suggests that when oxygen is present, metabolism will generally follow oxidative processes, thus describing the O<sub>2</sub> driven suppression of glycolytic metabolism.**

#### **D.6 Aerobic glycolysis (Warburg effect)**

The Warburg effect is a unique metabolic situation where the Pasteur effect does not hold up. It can be understood through an examination of the historical findings by which it was discovered and defined. In the first half of the 1900's Otto Warburg observed a rapid increase in oxygen (O<sub>2</sub>) consumption accompanying cell division in sea urchin eggs upon fertilization[330]. Considering the rapid cell division associated with cancers he assumed he would find similar observations in rapidly

dividing tumor cells. However, upon measuring oxygen consumption rates and lactate production (from CO<sub>2</sub> measurements) in thin sections of Flexner-Jobling rat liver carcinoma with rapidly dividing cells he found that they did not demonstrate increased oxygen consumption compared to normal tissue but rather demonstrated increased lactate production in the presence of normal concentrations O<sub>2</sub> [331], a stark contradiction to the Pasteur effect. Further investigation found that approximately tenfold more glucose was metabolized than could be accounted for by oxidative metabolism [332], and the amount of lactic acid produced by cancer cells (from multiple carcinomas tested) was two orders of magnitude higher than that produced in normal tissue [333]. Warburg hypothesized that lactate production in normoxic conditions was due to some defect in normal respiratory processes making it unable to achieve the Pasteur effect and inhibit glycolytic energy production coupled to lactic acid fermentation [333].

It was later found (again by Warburg) that ascites tumor cells in culture behaved similar to the Flexner-Jobling tumor slices with elevated lactate production [334] and similar rates of respiration to yeast and muscle cells [335, 336]. This data somewhat modified Warburg's hypothesis of a breakdown in oxidative processes but rather suggested that there was no defect in respiration and that lactate production was not at the cost of respiration. Indeed Cori and Cori demonstrated increased lactate content was coupled with decreased glucose content in venous blood from tumor laden tissue compared to normal tissue [337, 338]. Similarly, Warburg measured arterial and venous blood in healthy and Jansen's sarcoma laden tissue [339, 340]. He found a 2-17%

reduction in glucose content across normal tissue compared to a 40-70% reduction across the tumors. Healthy tissue produced no lactate whereas the tumors produced lactate on average with 66% of the glucose consumed. However, these lactate measurements were potentially lower than actual lactate production as tumors have demonstrated the ability to recycle the lactate they produce under aerobic conditions [341]. This makes in vitro measurements more indicative of changes in glucose metabolism at a cellular level than the complex microenvironment of tumors at a cellular level.

Warburg found that the Meyerhof equation (an index of oxygen's ability to inhibit lactic acid fermentation) for tumor cells produced an index similar to that of normal cells [331]. This indicated that O<sub>2</sub> consumption in cancer cells was similar to that in normal cells and oxygen sensing was most likely intact. However, given that they demonstrate increased lactate production, the Pasteur effect was not supported in the tumor cells. Warburg believed this had to be some defect in respiration, or that it was inadequate to prevent lactate production [342, 343], and that higher rates of respiration, if possible, would inhibit lactate production [344]. However, contrary to this hypothesis experimental data has shown that increased aerobic conditions in tumors demonstrate increased lactate metabolism [341].

More recent review of Warburg and his colleagues' work couple with more recent data provides a better understanding of the Warburg Effect mechanistically. Warburg and his colleagues demonstrated that in the time it takes cancer tissue under

normoxic conditions to completely metabolize one molecule of glucose to generate 36 molecules of ATP, an additional 20 molecules of ATP are generated from 10 glucose molecules being metabolized to lactate [333]. Under anoxic conditions, the cancer cells produce 26 molecules of ATP from 13 molecules of glucose over the same time period. Therefore in the same time it would take a normal cell exhibiting the Pasteur effect and strict aerobic metabolism to produce 36 ATP molecules from one molecule of glucose, the anoxic cancer cell strictly relying upon glycolysis produces 26 ATP from 13 molecules of glucose, and the cancer cell exhibiting the Warburg effect (running both glycolytic and oxidative energy production) generates 56 ATP from 11 glucose molecules [282].

**This data demonstrates both oxidative and glycolytic metabolism production working in parallel in the Warburg effect as opposed to one inhibiting the other as assumed in the Pasteur effect.** Warburg and colleagues' own findings support this, their data demonstrating 46 milligrams of lactate production for 70 milligrams of glucose removed across tumors per 100 ml blood measured. This demonstrates a 10% increase in ATP production associated with aerobic glycolysis compared to normal non-tumor burdened tissue [339, 340]. More recently this has been confirmed by studies in LN18 glioblastoma cells which demonstrate a 13% increase in ATP production associated with increased lactate production [345].

#### **D.7 “Benefits” of Warburg Effect**

Tumor cells demonstrating this metabolic phenotype have sufficient ATP production as demonstrated by high ratios of ATP:ADP and NADH/NAD<sup>+</sup> [346, 347].

However, the tremendous inefficiency in production of only 2 ATP from glycolytic metabolism compared to 36 ATP from oxidative metabolism of one glucose molecule is remarkable. Why would the Pasteur Effect in which oxidative metabolism is proposed to dominate whenever conditions are permissive, not be the case for all cells? Why would this change for tumor cells? There are a number of reasons that have been proposed and identified using tumor cells as a model for study.

This process, though inefficient, provides beneficial effects for cancer cells in that it allows for biomass generation through the utilization of glycolytic intermediates [348, 349]. Proliferating cells require the ability to perform multiple anabolic events to replicate cellular contents prior to cytokinesis. This imposes a large need for lipids, amino acids, nucleotides, etc. While synthesis of these cellular components requires energy (in the form of ATP), it also poses additional requirements for generating building blocks for the synthetic pathways. As an example, generating nucleotides and amino acids uses more building block units (carbon, etc) than energy units (ATP); the yield from one molecule of glucose could contribute up to 36 ATP in energy generation or up to 6 carbons for molecular synthesis [284]. By performing aerobic glycolysis, glucose is not completely catabolized to CO<sub>2</sub>, instead the glycolytic intermediates are more available to be siphoned off for macromolecule synthesis. This is particularly important for adipocytes which need to generate three carbon glycerol backbones (glyceroneogenesis) for esterification and storage of fatty acids as triacylglycerol (will be discussed further under heading PDK).

## D.8 Aerobic glycolysis summary

The Warburg Effect or aerobic glycolysis is a unique metabolic phenotype whereby the Pasteur effect is not observed; but instead aerobic conditions are accompanied by increased lactate production. As described in tumor models, there is no decrease in oxidative processes and no defect in respiration, just an increase in lactate production (lactic acid fermentation) even in the presence of oxygen. Though inefficient from the standpoint of ATP yield per glucose molecule, it is sufficient for the ATP and macromolecule synthesis needs of rapidly dividing tumor cells. While lactate production has been demonstrated in human adipose tissue, why this lactate is produced at a cellular level has not been sufficiently explored. It is possible that adipocytes produce lactate in oxygen-replete conditions, similar to tumor cells. **This dissertation identifies the existence of a Warburg-like phenotype in differentiated human adipocytes and attempts to determine its regulation and purpose in these cells.**

## E. Metabolic ROS

### E.1 Introduction

Living in an oxygenated environment and depending on oxygen for energy-producing respiratory processes leads to the inevitable production of reactive oxygen species (ROS). Endogenous production of oxygen radicals was unheard of until Denham



Harmon introduced his 'free radical theory' in the 1950's [350]. This stated that cellular generation of reactive oxygen species did in fact occur, and resulted in cellular damage when it accumulated. While controversial, endogenous oxygen radical production gained more support nearly a decade later with the identification of superoxide dismutase (SOD) which functioned in aerobic organisms to remove superoxide anions [351, 352]. Since this seminal work, ROS production and regulation has been intensely studied. ROS can be both beneficial and detrimental to normal cellular processes, and dysregulation of ROS homeostasis has been implicated in multiple disease states. Reviewed here are the cellular sources of ROS and some of the mechanisms counteracting ROS production, some of the benefits and detriments of ROS production in cells, and its role in adipocytes.

## **E.2 ROS production**

There are multiple reactive oxygen species which can be generated exogenously or endogenously. Common examples of such ROS include hydrogen peroxide, superoxide anions, and hydroxyl radicals. These can be generated in the cytosol, primarily by NADPH oxidases. While initially described in neutrophils, NADPH oxidases have more recently demonstrated broad expression in multiple tissue and cell types and are involved in cellular transformation as well as replicative senescence [353-358] along with the historically-described phagocytic activity. There are a host of other enzymes for which normal function can result in ROS generation including but not limited to p450 enzymes, xanthine oxidase, lipoxygenases, and cyclooxygenases [359].

The bulk of cellular ROS is generated metabolically in the mitochondria. Mitochondria-derived ROS contribution to cellular damage, and mitochondrial ROS-based signaling to the rest of the cell have been well documented and reviewed [360, 361]. ROS production by the respiratory chain was initially reported in 1966 [362] followed by identification of  $H_2O_2$  production in isolated mitochondria [363-365] which was found to be a dismutation of superoxide [366, 367]. Some evidence exists indicating that mitochondria convert between 1-2% of consumed oxygen molecules into superoxide anions [363]. Mitochondrial ROS production is mostly generated by two components of the electron transport chain.

The electron transport chain involves the donation of electrons from electron donors (NADH, FADH<sub>2</sub>) through a series of complexes, eventually reducing oxygen to form water. Under normal circumstances with the appropriate reduction, four protons and four electrons are transferred to oxygen generating water in a harmless reaction. The rapid transport of electrons can allow for electron leakage generating superoxide radicals. While this can occur at several points along the electron transport chain, the two points that are especially susceptible to ROS generation are complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase). Production of ROS from these two sites is thoroughly detailed by Michael Murphy [368]. Briefly, complex I demonstrates elevated superoxide production in two basic situations. First, a reduced state of flavin mononucleotide (FMN) brought on by a high ratio of matrix NADH/NAD<sup>+</sup> (which essentially increases the rate of electron transport), and second, a situation where reverse electron transport occurs from electron transfer to Coenzyme Q

(CoQ) with an elevated proton-motive force. Complex III can generate a significant amount of ROS as it funnels electrons from the CoQ pool to cytochrome *c*. The free radical semiquinone anion (a component for regenerating coenzyme Q) readily transfers electrons to oxygen forming superoxide. This site is considered to be the primary point of reactive oxygen species production under baseline proton motive force [369] whereas complex I is considered the primary site of ROS production with elevated proton motive force [368].

Production of ROS within cells correlates with electron transport and therefore is heavily dependent upon the rate of oxidative phosphorylation providing the necessary NADH and FADH<sub>2</sub> to drive electron transport. Essentially, the processes of the electron transport chain are the primary source of metabolic ROS. When the processes that drive oxidative phosphorylation are elevated (elevated rates of the electron transport chain generating a high proton motive force), more ROS is produced.

### **E.3 ROS Regulatory Role and Stress**

While originally viewed as detrimental to normal cellular function (along with phagocytic function in leukocytes) a critical role for normal cell signaling has emerged for ROS. As an example, PDGF or EGF signaling stimulate a burst of ROS production upon receptor activation [370, 371] that targets associated phosphatase activities to regulating receptor tyrosine kinase signal transduction [372, 373]. The field studying ROS associated signaling has expanded in recent years with technologic advances in broad spectrum proteomics and computational analysis capable of detecting

nitrosylation and glutathionylation of signaling proteins as a result of ROS post-translational modification [374, 375]. While ROS signaling was initially associated with membrane and cytosolic ROS generation, a number of cellular processes have been identified as being regulated by mitochondrial-derived ROS. These include regulating HIF-1 $\alpha$  in hypoxia [376-378], response to metabolic excess [379], regulation of Atg4 dependent autophagy [380], and inflammatory response [381-383].

The degree of cellular reactive oxygen stress is determined by the amount of reactive oxygen species production and eliminated. Severe stress occurs when accumulation occurs either by excess production or deficiency of defenses against excess ROS accumulation. A buildup of ROS can cause damage to normal cellular processes including DNA damage, protein damage, misfolded protein response, and lipid peroxidation all of which can lead to altered normal cellular function and even cellular necrosis or apoptosis.

#### **E.4 ROS in Adipocytes**

In adipocytes, ROS have demonstrated a regulatory effect on adipocyte differentiation. Multiple signaling factors sensitive to ROS modulation have been implicated in this regulation including CHOP, mTORC1, FOXO1, TNF $\alpha$ , and Nrf2 among others [384-388]. Under normal conditions ROS signaling has a role in regulation of adipogenic differentiation induction and also in directing differentiated cells to terminal adipocyte differentiation [388-390]. However, under various conditions, ROS role in regulating adipogenic differentiation is altered. For example, in elderly individuals

normal adipogenic differentiation is suppressed [387, 391, 392], a process that is heavily influenced by ROS regulated-CHOP signaling [387, 393-395].

Furthermore normal adipocyte function is altered by dysregulation of normal ROS production resulting in oxidative stress. Along with the direct insults of reactive oxygen stress already discussed (DNA and protein damage, apoptosis, necrosis, etc.) a number of cellular alterations have been described in adipocytes in response to oxidative stress including unfolded protein response (UPR) [396], alterations in adipokine production [397-403], and an inflammatory response [396, 403-406]. Together, these alterations in normal cellular physiology contribute to disease states. For example, oxidative stress in adipocytes is associated with the metabolic syndrome, causes insulin resistance and further progression into type II diabetes [398, 403, 407-414]. Other less studied diseases associated with oxidative stress in adipose tissue include chronic kidney disease [406], atherosclerosis [400], arthritis [415], and sleep apnea [416]. These disease state correlations demonstrate the importance of regulation of ROS production in adipocytes for maintenance of normal whole animal/human physiology.

### **E.5 Aerobic glycolysis as a protection against damaging ROS**

Tumor cells demonstrate a high degree of ROS and require careful mechanisms to keep ROS in balance to prevent reactive oxygen stress mediated damage [417]. As for most cells, the terminal steps of aerobic metabolism (the electron transport chain) is the primary source of metabolically-derived ROS in tumor cells. Many tumor cells

reduce ROS generation rates by increasing the fraction of ATP generated through glycolytic metabolism in the cytosol as opposed to ROS-producing oxidative metabolism in the mitochondria [418]. Essentially by employing this (Warburg effect/aerobic glycolysis), they limit overproduction of ROS reducing detrimental reactive oxygen stress. This has been validated in a number of human clinical trials with therapeutic mechanisms of preventing tumor cell aerobic glycolysis and its associated cytoprotective effects against ROS over-production resulting in tumor cell death. A number of suspected regulators of aerobic glycolysis have been targeted to induce this ROS mediated tumor cell cytolethality including PKM2 [419, 420], LDHA [285, 421, 422], and the PDKs [423-429]. This demonstrates that purposeful alterations in cellular metabolism (upregulation of aerobic glycolysis) can be employed by cells as cytoprotection against detrimental reactive oxygen stress.

## **E.6 Metabolic ROS summary**

Reactive oxygen species are primarily generated metabolically through the terminal steps oxidative phosphorylation in the electron transport chain. These ROS can serve a beneficial signaling role regulating multiple cellular processes, but dysregulated overproduction of ROS can be detrimental to normal cellular function and even lead to cell death. In adipose tissue, ROS have demonstrated a role in regulating cellular function, but when dysregulated, reactive oxygen stress contributes to cellular dysfunction and multiple disease states. This suggests that regulation of ROS (limiting overproduction of ROS) critical for normal adipocyte function. Tumor cells have

demonstrated the ability to switch to a metabolic phenotype characterized by elevated lactate production from the Warburg Effect or aerobic glycolysis. In these cells, this regulates reactive oxygen stress for cytoprotective purposes. Adipose tissue has also demonstrated the ability to produce lactate, and though it has not been explored, perhaps lactate production from adipocytes is the result of an ability to alter their metabolic phenotype to aerobic glycolysis as a protective mechanism against over production of ROS. **This dissertation attempts to demonstrate a potential role of a Warburg-like phenotype as a protective mechanism regulated in adipocytes against detrimental production of ROS.**

## **F. PDKs**

### **F.1 Intro**

The PDKs (pyruvate dehydrogenase kinases) are a series of enzymes that regulate oxidative and glycolytic metabolism with tissue dependent differential expression. As a whole they function to decrease the amount of oxidative metabolism by reducing the activity of the pyruvate dehydrogenase complex. They have been extensively studied in terms of regulation with some roles in adipose tissue for macromolecule generation. A number of pharmacologic regulators have been identified that have been used in studies with tumor cells identifying a role for the PDKs in regulating the Warburg effect in these models.

## **F.2 Pyruvate dehydrogenase complex and PDK function**

The “selection” between executing glycolytic (glycolysis coupled with lactic acid fermentation) and oxidative metabolism is dependent upon the fate of pyruvate, after pyruvate is generated through the oxygen-independent breakdown of glucose through glycolysis. Pyruvate can be turned into lactate through an oxygen-independent method (lactic acid fermentation) only generating energy from the ATP generated in the payoff phase of glycolysis or it can be further oxidized (oxidative phosphorylation) through conversion to acetyl-Coenzyme A and transport into the mitochondria fueling the TCA cycle generating energy by powering the electron transport chain through NADH and FADH<sub>2</sub> production. The balance of how much glycolytic and oxidative metabolism a cell runs depends primarily upon regulation of pyruvate’s conversion to acetyl-Coenzyme A. With slower conversion to acetyl-CoA even in normoxic conditions, pyruvate accumulates and is converted to lactate increasing the cell’s fraction of energy generation from glycolytic metabolism. When acetyl-CoA is rapidly formed, pyruvate is less likely to be converted to lactate and the cell’s fraction of energy generation from oxidative metabolism increases.

The formation of acetyl-CoA from pyruvate is achieved by the pyruvate dehydrogenase complex (PDC). PDC regulates the transformation of pyruvate into acetyl-CoA producing NADH in the process. As a member of the 2-oxo acid dehydrogenase complexes, PDC is a multienzyme complex with copies of three enzymes; these are termed E1, E2, and E3. E1 is considered a 2-oxo acid



dehydrogenase; E2 is a dihydrolipoamide acetyl transferase; and E3 is an FAD-containing dihydrolipoamide dehydrogenase; these subunits assemble in the mitochondria [430-432]. The E1 complex contains 30 tetrameric pyruvate dehydrogenase (PDH) components, the E2 subunit which binds to E1 is made of a 60-meric dihydrolipoamide acetyl transferase, which is also bound to the E3 subunit containing multiple copies of homodimeric dihydrolipoamide dehydrogenase [430, 432]. Acetyl groups from the E2 subunit of PDC are transferred onto oxidatively decarboxylated pyruvate (action from the E1 subunit) forming acetyl-CoA [430]. This formation of acetyl-CoA is irreversible.

PDC activity is regulated by the phosphorylation state of PDH demonstrating inactivation by phosphorylation. There are four pyruvate dehydrogenase kinases (PDK) that actively phosphorylate PDC and two pyruvate dehydrogenase phosphatases (PDP) dephosphorylating PDC that are essentially responsible for regulation of the phosphorylation state of PDH. The PDH PDPs are phosphoprotein phosphatase 2C family members [430, 433, 434]. Of these two phosphatases, PDP1 is primarily expressed in muscle, but also detected in heart and brain; while PDP2 is primarily found in adipose tissue but also expressed in kidney, brain, and heart [430, 433, 435].

This section will focus on the activity of the PDKs. The PDKs bind to lipoyl domains of E2 and E3 binding protein (E3BP) to gain access to the phosphorylation sites on the E1  $\alpha$  component where they regulate PDH and subsequently PDC by site-specific phosphorylation [430, 436, 437]. There are three sites on PDH that are phosphorylated: Ser264 (site 1), Ser271 (site 2), and Ser203 (site 3). As the pyruvate dehydrogenase

(PDH) subunit of PDC exists as a heterotetramer ( $\alpha_2\beta_2$ ), there are six sites available on PDH for phosphorylation. Although phosphorylation at any one of the sites is sufficient suppress PDC activity [438-441], the mechanism of inhibition is different at each site with the majority of phosphorylation occurring at site 1 compared to sites 2 and 3 [442].

### **F.3 Differential expression and activities of PDKs**

There are five mitochondrial protein kinases encoded in eukaryotic cells. Four of the five are the PDKs have already been mentioned. The final protein kinase in all eukaryotic cells is a branched-chain  $\alpha$ -keto acid dehydrogenase [430, 443-445]. In mammals, the four PDKs demonstrate differential expression in different tissues [430, 435, 446]. PDK1 is present in the heart with lower levels in pancreas, liver and muscle. PDK2 is more ubiquitously expressed being found in many tissues. And PDK3 is found mostly in testis and to a lesser extent in the kidneys, brain, and lungs, and PDK4 is highly expressed in skeletal muscle and heart with variable levels in lung, liver and kidney.

To determine the activities of each of the four PDKs, PDC was reconstituted with specific mutagenesis at two of the three phosphorylation sites (alanine substitution) to allow phosphorylation of only one functional serine (one site) [447]. These studies demonstrated that all four PDKs can phosphorylate sites one and two ( $\text{Ser}^{293}$ ,  $\text{Ser}^{300}$ ), while only PDK1 phosphorylates site three of PDH ( $\text{Ser}^{232}$ ,  $\text{Ser}^{293}$ ,  $\text{Ser}^{300}$ ). Differential rates of phosphorylation were observed for sites 1 and 2 for the different enzymes in phosphate buffered saline. For site 1:  $\text{PDK2} > \text{PDK4}, \text{PDK1} > \text{PDK3}$ ; for site 2:  $\text{PDK3} > \text{PDK4} > \text{PDK2} > \text{PDK1}$ . Site specific alterations in phosphorylation coupled with

differential tissue expression is most likely responsible for unique tissue regulation of PDC activity in both normal and pathophysiologic states [430, 435, 446, 447].

#### **F.4 Regulation of PDK expression**

PDC demonstrates both short-term and long-term regulation by phosphorylation. Rapid alterations in phosphorylation state and therefore activity of PDC are achieved by pyruvate dehydrogenase phosphatase (PDP1) (activated by  $\text{Ca}^{2+}$ ) and PDK (activated by acetyl-CoA and NADH and inhibited by pyruvate) [430, 433]. From rodent and in vitro studies, more long-term regulation of PDC has been proposed to be achieved by relative expression of PDKs in a given tissue under various physiologic and pathophysiologic conditions [430, 443, 448].

*In vivo* studies in rat have demonstrated long term regulation of PDC activity through alterations in PDK expression. PDKs 1,2 and 4 are expressed in rat heart with PDK 4 demonstrating increased expression associated with decreased PDC activity under starvation and diabetic conditions [430, 448-451]. Similarly, both diabetic state and starvation correlate with increased skeletal muscle levels of PDK4 and PDK2 (smaller increase) [430, 452, 453]. PDK2 expression increases as does PDK4 to a lesser extent preserving three carbon sources for gluconeogenesis under starvation in rat liver [430, 453-455]. Glucose production is also protected in the kidney during starvation by increased expression of PDK4 and PDK2 [430, 453, 456]. In lactating rats, PDK4 and PDK2 are increased with starvation most likely responsible for the inactivation of lipogenesis necessary for normal milk production [430, 457, 458]. All these studies

taken together demonstrate the critical role that has been described for PDKs (especially PDK4 and PDK2) in regulating response to starvation and in the diabetic state. Contrary to what has been observed in the above tissues, starvation demonstrated no effect on PDK2 and PDK4 protein expression (though a two fold increase in message was observed in PDK4) [453] with no increase in PDK activity in brain [459]. However, from these studies it is difficult to determine if minute subpopulations of neurons may demonstrate response to starvation.

A number of ligands have demonstrated regulation of PDK expression as well. Treatment of rats with the stable fatty acid surrogate WY-14,643 elicits an increase in PDK activity and PDK4 expression in skeletal muscle and liver through PPAR $\alpha$  dependent mechanisms [430]. Similar findings were observed in vitro; after treating 7800C1 hepatoma cells with WY-14,643, PDK4 message significantly increased [430, 460]. In this cell line and in HepG2 cells, dexamethasone (glucocorticoid) treatment results in increased expression of PDK4 through a Protein Kinase B- $\alpha$  mechanism [430, 461]. Consistent with expectation based on effects of diabetic and fasted states (increased PDK), insulin and refeeding demonstrate reversal of the increased PDK4 expression associated with those metabolic and disease states [430, 451, 453]. Similarly, insulin antagonizes the dexamethasone effect reducing dexamethasone stimulated increases in PDK4 and effectively reduces PDK2 expression in the presence of both WY-14,643 and dexamethasone [430, 461].

PDK regulation of PDC (and associated PDP regulation) are thought to also play a critical role in regulating blood glucose, this has been reviewed by Harris et al [462]. In the well-fed state, directing carbon from pyruvate to the citric acid cycle by highly active PDC helps to reduce total blood glucose. Similarly, in low-glucose states, the reduction of PDC activity (by PDK action) conserves pyruvate for gluconeogenic activities in the liver. These hypotheses are increasing the interest in pharmacologic regulation of PDK as a potential target for diabetic care. Supporting the therapeutic potential of PDK modulation, PDK4 knockout in animals demonstrated lowered fasting glucose [463], improved performance in glucose tolerance tests [464], and reduced negative effects of long-term high saturated fat diet [465]. Similarly, interest has grown in the potential association of PDK4 polymorphisms with diabetes, though further studies with larger cohorts are most likely required to definitively identify strong correlations between the identified single nucleotide polymorphisms (SNPs) and diabetes [466].

#### **F.5 PDKs in adipose tissue.**

Studies examining the effects of starvation on white adipose tissue demonstrated no increase in PDK2 or PDK4 protein [467]. However, four-fold increase in PDK4 message is observed in white adipose in response to starvation [467]. In adipose tissue, PDP plays a role in the short term regulation of PDC activity in an insulin dependent method [468, 469] which may be sufficient for response to starvation and the decrease in systemic insulin levels. Longer term starvation studies have not been reported but may perhaps demonstrate significant changes in PDK4 expression in white

adipose tissue as a mechanism of long-term regulation. In 3T3-L1 adipocytes both prolactin and growth hormone treatment increased PDK4 expression in a STAT5 dependent manner [470]. In parallel 3T3-L1 studies, the elevated PDK4 associated with growth hormone and prolactin treatment decreased insulin stimulated glucose uptake [470] and has been proposed as a contributor to prolactin-induced insulin resistance.

In white adipose tissue lipolysis and free fatty acid re-esterification to glycerol are critical for regulating non-esterified fatty acid (NEFA) release and uptake/storage requiring glycerol as a substrate for such reactions [471, 472]. This requires synthesis of glycerol-3-phosphate (G3P) mostly generated from lactate and pyruvate through glyceroneogenesis [473, 474]. As this pathway requires lactate and/or pyruvate as substrate, PDK inhibition of the PDC conversion of pyruvate to Acetyl-CoA (decreasing PDC activity) benefits glyceroneogenesis. Accordingly, human white adipose tissue explants and 3T3-F442A adipocytes treated with rosiglitazone (PPAR $\gamma$  agonist) demonstrated increases in PDK4 associated with increased rates of glyceroneogenesis [471]. Inhibition of PDK activity with pharmacologic inhibitors leelamine and dichloroacetic acid (DCA) altered NEFA release in both basal and rosiglitazone treated states indicating PDK4's role in glyceroneogenesis in adipocytes.

## **F.6 Pharmacologic regulation of PDKs**

Leelamine has been identified as a relatively specific small molecule inhibitor of PDKs [475]. Treatment causes PDK inhibition resulting in decreased glyceroneogenesis in adipocytes [471] and lowers blood glucose in *ob/ob* mice [475]. Thus, leelamine has

emerged as one of the leading candidates for the potential treatment of hyperglycemia by targeting PDKs [462] and as a tool for modulating PDK activity in vitro and ex vivo [471]. Similarly, dichloroacetate (DCA) has demonstrated PDK inhibition and lowers blood glucose [476-478]. 2-chloropropionate and  $\alpha$ -lipoic acid both inhibit PDKs and have demonstrated some level of lowering blood glucose [479-481]. Other small molecule PDK inhibitors have been synthesized by pharmaceutical companies with various potencies against the different PDKs in hopes of generating small molecule therapies for diabetes. These include SDZ048-619 and AZD7545-43 which have demonstrated decreased gluconeogenesis in liver and enhanced oxidative capacity in skeletal muscle [482-485].

#### **F.7 Cytoprotective role of PDKs in regulating aerobic glycolysis and ROS production in cancer cells**

As previously reviewed, aerobic glycolysis or an increased production of lactate without a change in oxygen tension has been most clearly described in cancer cells. As previously discussed, this confers some level of survival or resistance to apoptosis through reduction of metabolically produced ROS. The aerobic glycolysis phenotype has been considered to be regulated primarily by HIF-1 $\alpha$  and its molecular targets. More recently PDKs have been identified as one of the key downstream regulators of the concurrently increased lactate production and associated decreased ROS in tumor cells, conferring cytoprotection [486, 487]. Multiple studies in cancer cells have confirmed the PDK regulation of aerobic glycolysis in cancer cells. Overexpression of a less active

PDK leads to decreased lactate production, increased ROS production, cell death, and failure of tumor formation in xenograft models [488]. Similarly, pharmacologic inhibition of PDK in multiple cancer cell lines A549, M059K, and MCF-7 resulted in decreased lactate production, increased ROS and apoptosis [489]. In another study, pharmacologic inhibition of PDK resulted in a reversal of glycolytic phenotype, decreased proliferation rate, increased apoptosis, and reduced tumor growth in multiple breast cancer models [490]. In head and neck squamous cancer (HNSCC), PDK1 was found to be elevated in human tumor biopsies compared to normal tissue and was more predominantly expressed in patients who demonstrated local recurrence and metastasis [491]. Studies on HNSCC lines demonstrated decreased lactate production and decreased proliferation rates as well [491]. These findings have led to PDKs as an interesting target for tumor therapy with a mechanism to remove the cytoprotective role of aerobic glycolysis in cancer cells [492, 493]. Altogether this body of research demonstrates the central role of PDKs as key regulators of aerobic glycolysis.

## **F.8 PDKs summary**

With the well-defined function of PDKs in regulating oxidative and glycolytic metabolism and their expression in adipocytes it is possible that they play a role in regulating metabolic phenotype in this tissue. Their role in cancer cells suggests that they could regulate lactate production in adipocytes as well. This dissertation will examine these possibilities using expression studies of PDKs in undifferentiated and differentiated cells in which metabolic phenotype shifts are observed. Using the



previously discovered pharmacologic inhibitors, the potential regulatory role of PDKs in adipocyte metabolism will be further dissected.

#### **G. Summary of Introduction and Statement of Hypothesis**

With the broad pathophysiologies and co-morbidities associated with obesity and its epidemic proportions, a better understand of adipose tissue and more importantly adipocyte biology has emerged as a critical area of physiologic studies. While lactate production has been observed in adipose tissue, how this occurs at a cellular level, the utility of lactate production by the adipocyte, and how this is regulated are not understood. Our central hypothesis is that differentiated adipocytes demonstrate a PDK-regulated Warburg-like effect protecting against detrimental elevation of ROS.

## **Chapter II:**

### **Results**

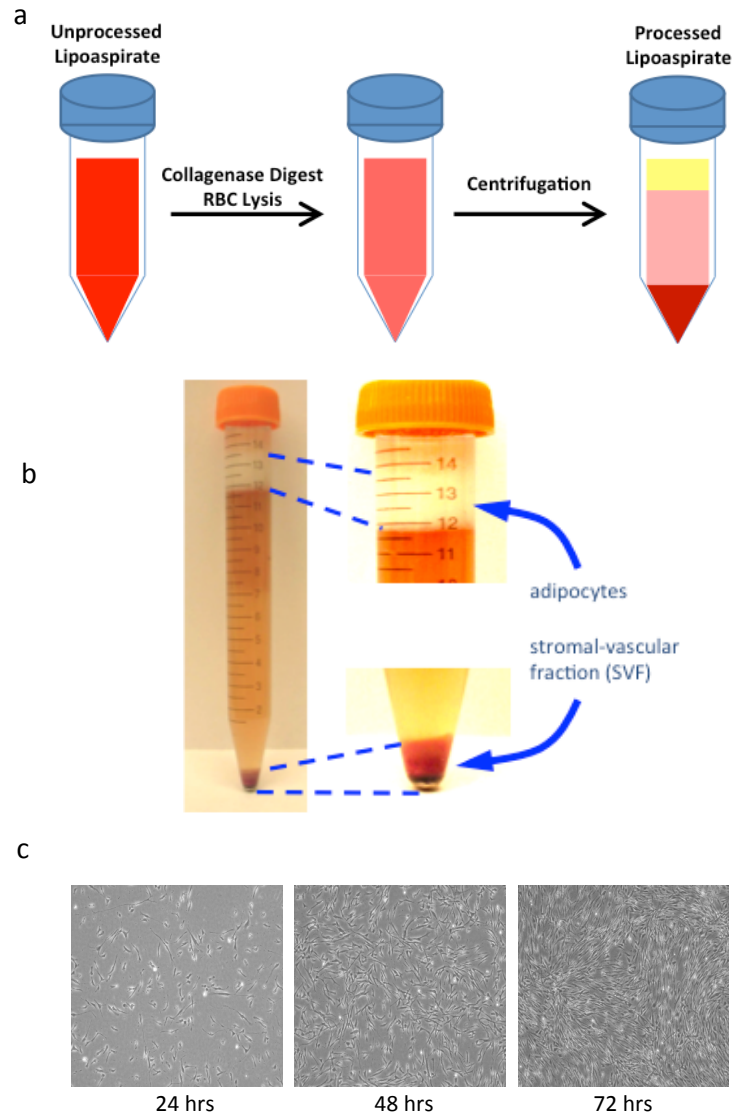
#### **A. Adipogenic Model**

To understand the metabolic phenotype of human adipocytes we optimized and characterized a model of human adipogenic differentiation starting with human adipocyte precursors (ASCs) from the stromal vascular fraction of human adipose tissue. Substantial time and effort was put into this model to be sure we had appropriate differentiation and complete differentiation so that inferences made about metabolism of adipocytes (especially compared to their preadipocyte precursors) would be as reliable and predictive of human adipocyte biology as possible when using a differentiation model to emulate the mature cells.

##### **A.1 Isolation and culture of human preadipocytes**

To study adipocyte metabolism, human preadipocytes were isolated from the stromal vascular fraction of lipoaspirate and differentiated into mature adipocytes. Liposuction is a procedure in which patients undergo anesthesia either as intravenous sedation or general anesthesia followed by infusion into the adipose tissue and loosening of excess fat using a cannula inserted through small incisions. The dislodged fat is suctioned out of the body using surgical vacuum or syringe attached to the

cannula. The dislodged tissue or lipoaspirate is usually discarded, but it can serve as a large source of ASCs. Through collagenase digestion the unprocessed lipoaspirate is rendered into a primarily single cell suspension. Subsequent red blood cell lysis and centrifugation separates the more buoyant adipocytes from the stromal vascular fraction (SVF) which pellets at the bottom of the tube. (Fig. 1a). These two populations are visibly separate in conical tubes after centrifugation (Fig. 1b). The SVF after plating on tissue culture treated plastic obtains a fibroblast like appearance. The cells proliferate rapidly, maintaining a spindle-like fibroblast morphology when plated in media containing various growth factors (Fig. 1c).



**Figure 1. Isolation of ASCs.** Isolation of ASCs from lipoaspirate or minced abdominoplasty tissue general flow scheme a. After digestion and centrifugation an image of separated adipocytes and the stromal vascular fraction b. After isolation and culture in T flasks images were taken at 24, 48, and 72 hours c.

## A.2 Cellular homogeneity

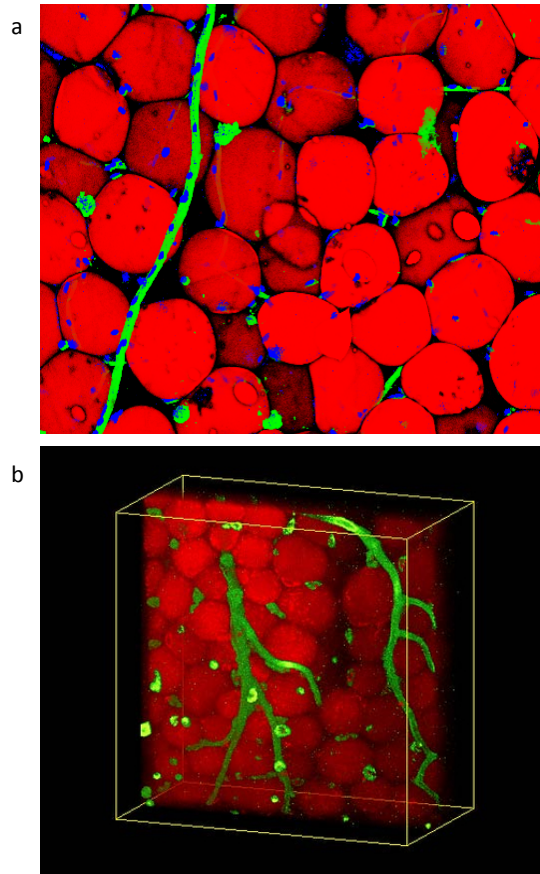
As with any model of differentiation it is important that the cellular material used for differentiation is well-defined, that is an understanding of what is being differentiated is critical to controlling the experiment. This becomes more complicated when the starting material for differentiation is primary isolated cells from tissue. In modeling human adipogenic differentiation to obtain primary differentiated adipocytes, the beginning material comes from the previously-described SVF. This contains contaminating cell types (non adipocyte precursors) that can alter final differentiated populations. It is critical that we demonstrate the potential contaminating cell types associated with isolation of the adipose stromal vascular fraction are essentially absent from our differentiation model. By doing this we can be more confident of our findings associated with differentiation and adipocyte metabolism.

Similar to what has been described in mice, confocal microscopy of isolated human subcutaneous adipose tissue demonstrates a high degree of vasculature (Figure 2a, b). This can contribute endothelial cells to the SVF as a contaminating population. Similarly, it is well documented that there is potential for a large leukocyte population (primarily macrophage) in adipose tissue that can also contribute to non-adipocyte precursors in the SVF. Both the endothelial cells and leukocytes (macrophage) along with the ASCs collect in the SVF from adipose tissue while the adipocytes remain in the more buoyant adipocyte fraction (Figure 3) generating the potential for contaminating populations in primary ASC isolation. With this potential contamination in the SVF we

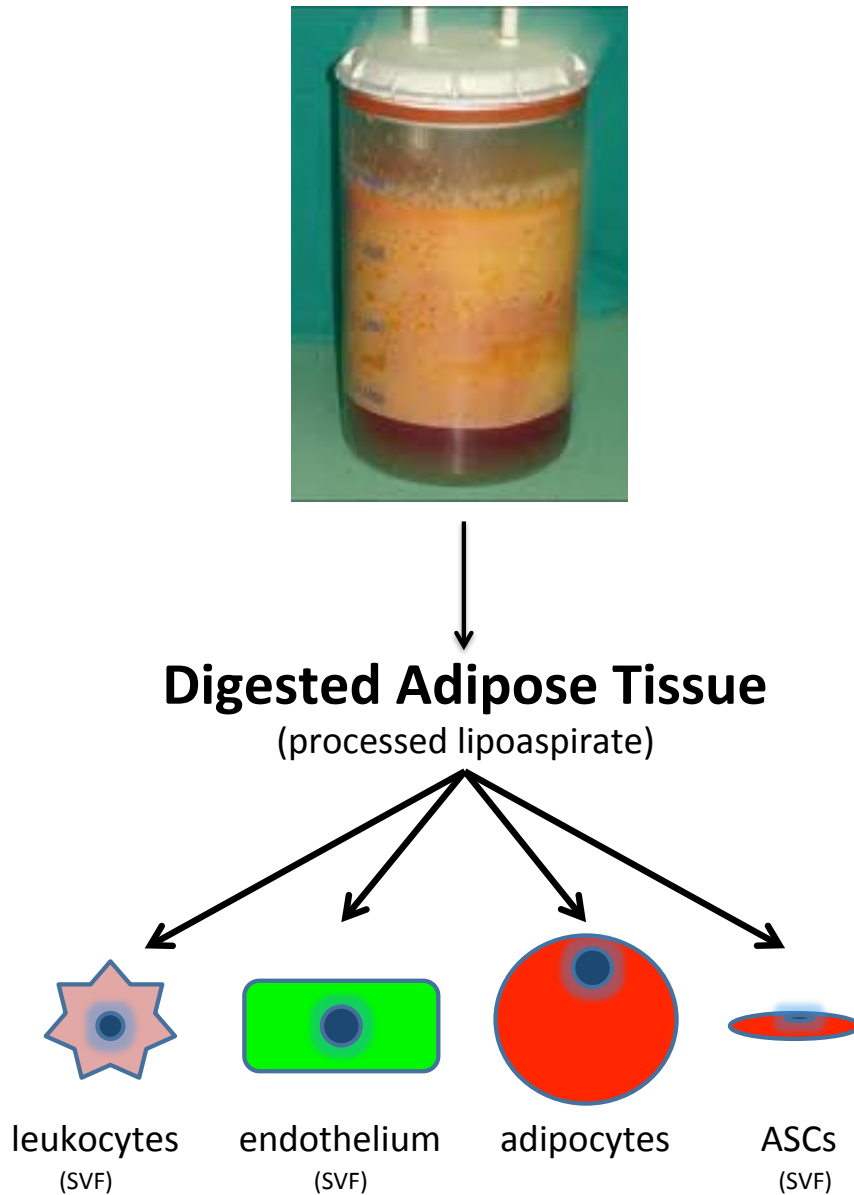
examined the cell cultures we used for adipocyte differentiation for the existence of these potential contaminating populations by flow cytometry after cell expansion. Analysis of several primary isolations and a control cell line showed culture-expanded ASC populations to be negative for the leukocyte marker CD45+ (PTPRC, Protein Tyrosine Phosphatase, Receptor Type, C) (Fig. 4a) and the endothelial cell marker CD31+ (PECAM-1, Platelet/Endothelial Cell Adhesion Molecule) (Fig. 4b). While this data indicated our expanded population was negative for the potential contaminating populations, we further determined the homogeneity of the populations using a marker for which the adipocyte precursors should be positive, CD140b. Data indicated the ASCs were homogeneously positive for this marker (Fig. 4c). Human dermal Fibroblasts (HDF) from Lonza were tested in parallel as a control population. This is a commercial cell line that is devoid of endothelial cells and leukocytes and expresses the same positive marker as ASCs (CD140b) making it an excellent control for the ASCs to look for absence of contaminating populations and homogeneity for a positive marker. Flow cytometry indicated the ASCs appeared similar to HDF for lack of leukocyte and endothelial contamination and cellular homogeneity for the positive marker (Fig. 4d).

Taken together, the data indicate that our isolation and expansion technique results in a homogeneous population of ASCs devoid of potentially contaminating ECs and leukocytes. Homogeneity for the positive marker and absence of contaminating cell types was consistent across multiple donors. This data demonstrates the isolation/cell source is usable for modeling differentiation within a single donor (clear, homogeneous

starting material) and across multiple donors (similar starting material with regard to cell types regardless of isolation).

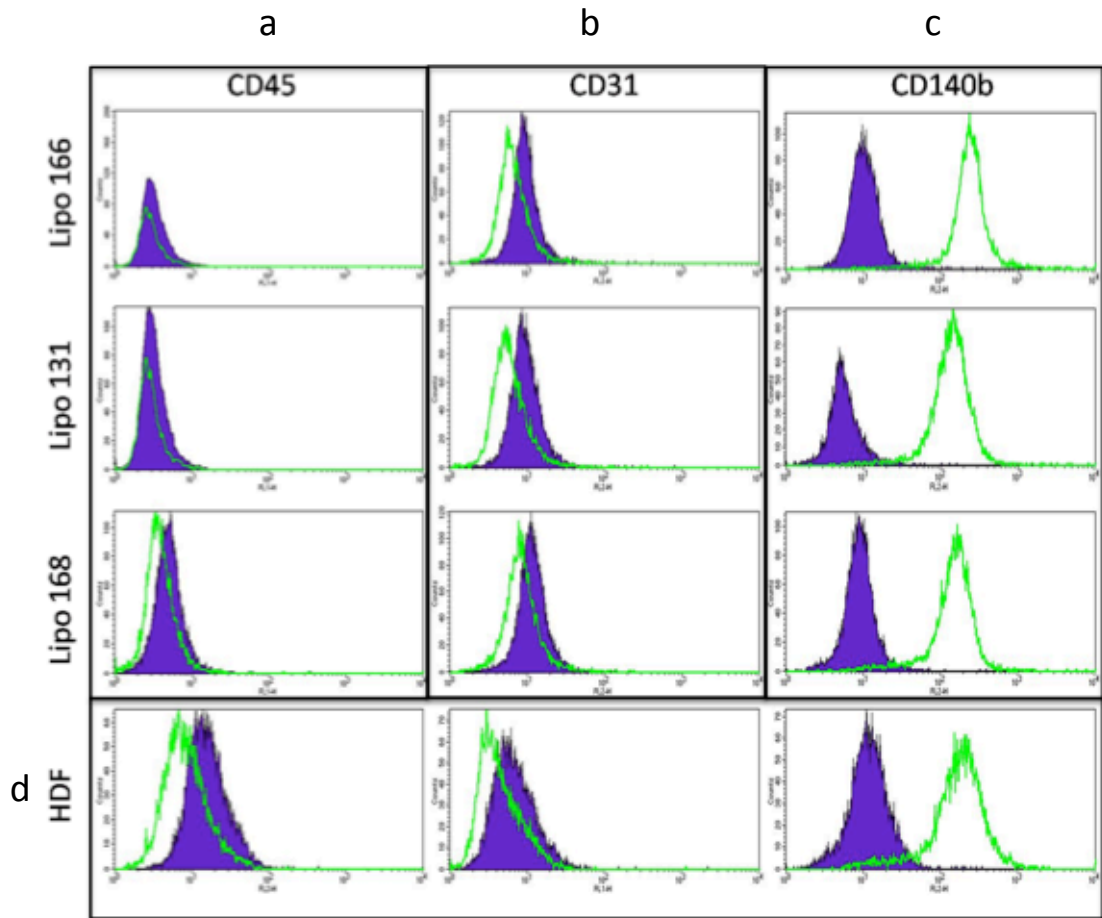


**Figure 2. Cellular structure of human adipose tissue.** Human subcutaneous adipose tissue was obtained from abdominoplasty. Small pieces of tissue were dissected away from the tissue and stained with Bodipy Red (red, lipophilic fluor), isolectin IB4-AlexaFluor 488 (green, vascular binding), and DAPI (blue, nuclei). Multiple planar images were captured using a multiphoton microscope demonstrating a high degree of vascularization (a). Planar sections were rebuilt into three dimensional representations using Amira software to better visualize vascular density in the tissue (b).



**Figure 3. Primary cell types abundant in processed lipoaspirate.** Image of unprocessed lipoaspirate, upon digestion and single cell separation processed lipoaspirate contains multiple primary cell types in abundance including leukocytes, endothelial cells, adipocytes, and ASCs. Such cell types can potentially contaminate preparations of ASCs.





**Figure 4. Cultured SVF is free of endothelial cells and leukocytes.** Expanded stromal vascular fractions of subcutaneous adipose tissue from three separate donors were expanded through passage 3 and subsequently analyzed by flow cytometry for presence of potential contaminating cell populations. In each histogram specific stain is shown in green with non-specific IgG in purple. Populations were stained as described in materials and methods for CD45 (a); CD31; and CD140b (c). In parallel, an established line of normal human dermal fibroblasts was run as a control(d).

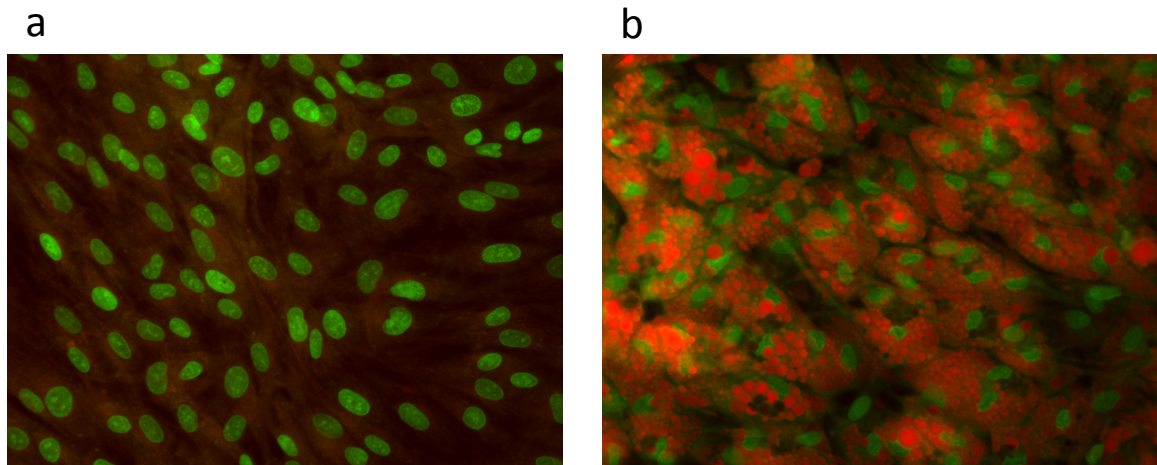
### A.3 Maintenance of post-expansion plasticity

With expansion of any primary cell there is the potential loss of phenotype. With cells that demonstrate some degree of cellular plasticity *ex vivo*, this plasticity is often one of the lost phenotypes. ASCs *in vivo* are proposed to have two potential differentiation lineages and mature functions or a bi-potentiality. The first and most classically defined lineage is formation of new adipocytes. More recently a second role (or potentially a second differentiation lineage) is associated with their occupying a perivascular niche and playing a pericytic role (either differentiating from a precursor into pericytes, or serving as pericytes prior to differentiation as a pool of undifferentiated adipocytes) [254, 494-496]. To determine whether or not our isolation and expansion caused loss of *in vivo* phenotype with respect to cellular plasticity or rather bi-potentiality we tested whether or not expanded ASCs could indeed differentiate down these lineages after the amount of primary cell expansion required to support our differentiation studies.

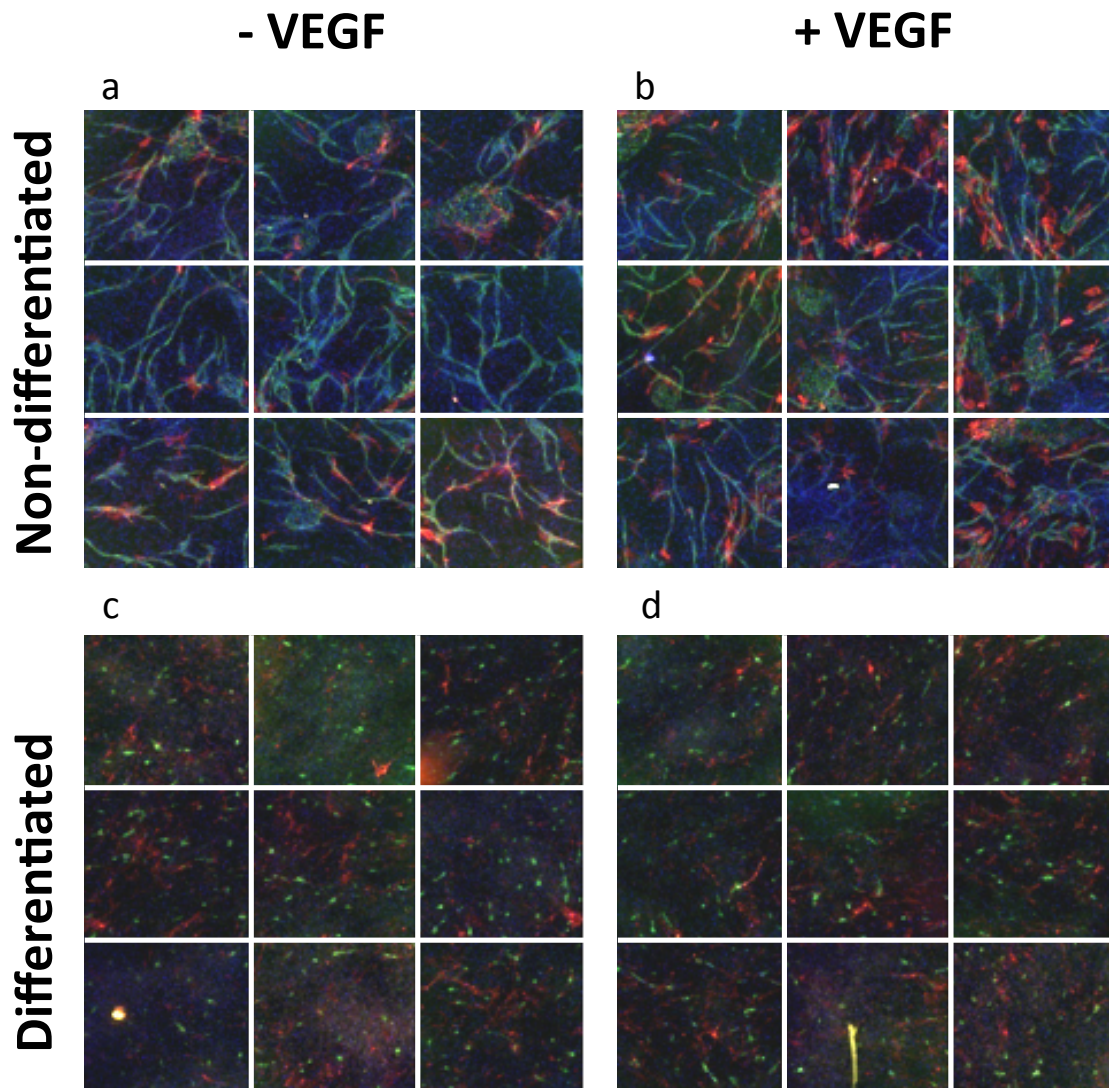
To evaluate the ability of ASCs to differentiate into adipocytes, we placed them under adipogenic differentiation conditions and stained the cells for lipid accumulation and morphometric changes. The cells demonstrated a high degree of adipogenic differentiation post-induction characterized by increased size and lipid droplet accumulation post differentiation (Fig 5b) as compared to undifferentiated ASCs (Fig 5a). These results indicated the cells maintained plasticity to differentiate down adipogenic lineage post expansion.

To determine whether the cells maintained a vasculosupportive role after expansion we co-cultured both undifferentiated and differentiated (adipogenic) with endothelial cells (HUVEC) to look for cord formation as an indication of fulfilling vasculosupportive role. Co-cultures were stained for cords (stained endothelial cells for CD31 to identify cord structures) and expression of the pericytic marker smooth muscle alpha actin. Cells not stimulated for adipogenic differentiation supported cord formation of the endothelial cells with ASCs in close proximity to the ECs demonstrating expression of smooth muscle alpha actin (Fig. 6a). Smooth muscle alpha actin expression appeared to be upregulated in response to VEGF (Fig. 6b) indicating that the cells contribute to a vascular system responding to physiologic vascular stimulus. The differentiated cells did support endothelial cell survival demonstrated by existence of CD31+ cells but did not support cord formation (Fig. 6c). The differentiated co-cultures did not respond to VEGF (Fig. 6d). This data indicated that the expanded cells do maintain the ability to support vasculature and potentially even to further differentiate into a more mature pericytic cell type (increased SMA expression).

Taken together the data from these differentiation studies indicate that the isolation and expansion procedure used for our studies does not result in a loss of the *in vivo* bi-potentiality of the ASCs. It also demonstrates that the cells can be used as tools to model both adipogenic differentiation and angiogenesis (cord formation support). This is evidence that our expanded cells are behaving physiologically with respect to differentiation capacity.



**Figure 5. Expanded ASCs maintain adipogenic lineage differentiation.** ASCs at passage 3 were differentiated as described in materials and methods. Cells were stained with Syto 16 (green, nuclear stain) and Nile Red (lipophilic red fluor) and imaged using widefield fluorescence. Cells were fixed and stained prior to (a) and after differentiation (b).

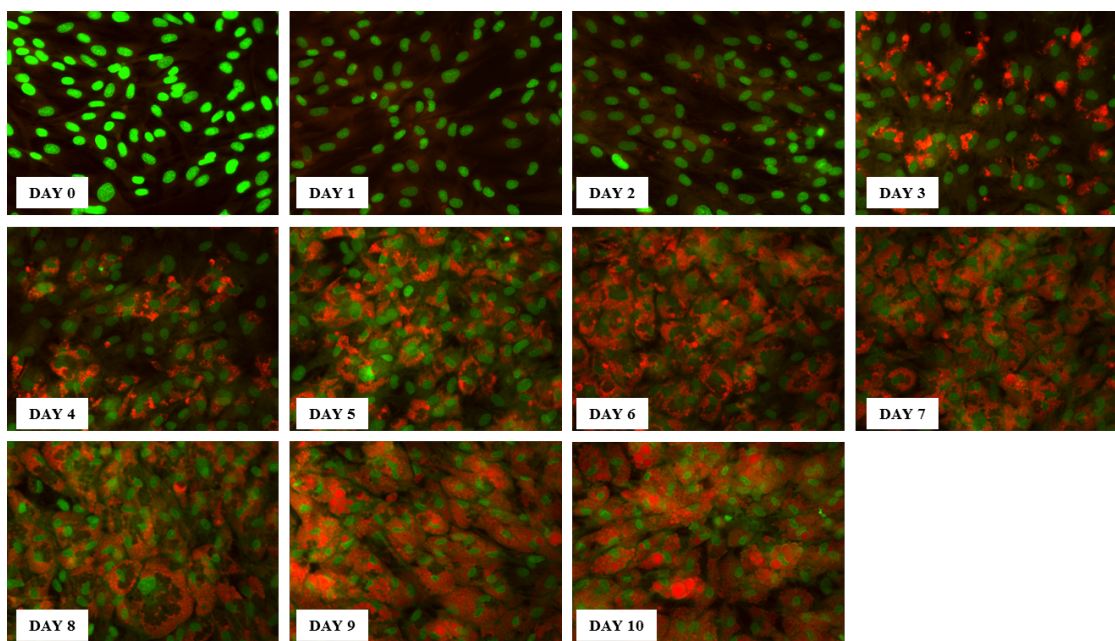


**Figure 6. Expanded ASCs maintain vasculosupportive phenotype.** ASCs at passage 3 were co-cultured with HUVECs for 3 days. After co-culture, cells were fixed and stained for CD31 (green) SMA (red) and dapi (blue, nuclei). Undifferentiated ASCs were cocultured with HUVECs (a) and cocultured in the presence of VEGF (b). Huvecs were also cocultured with differentiated ASCs both without and with VEGF (c, d).

#### **A.4 Quantifying differentiation**

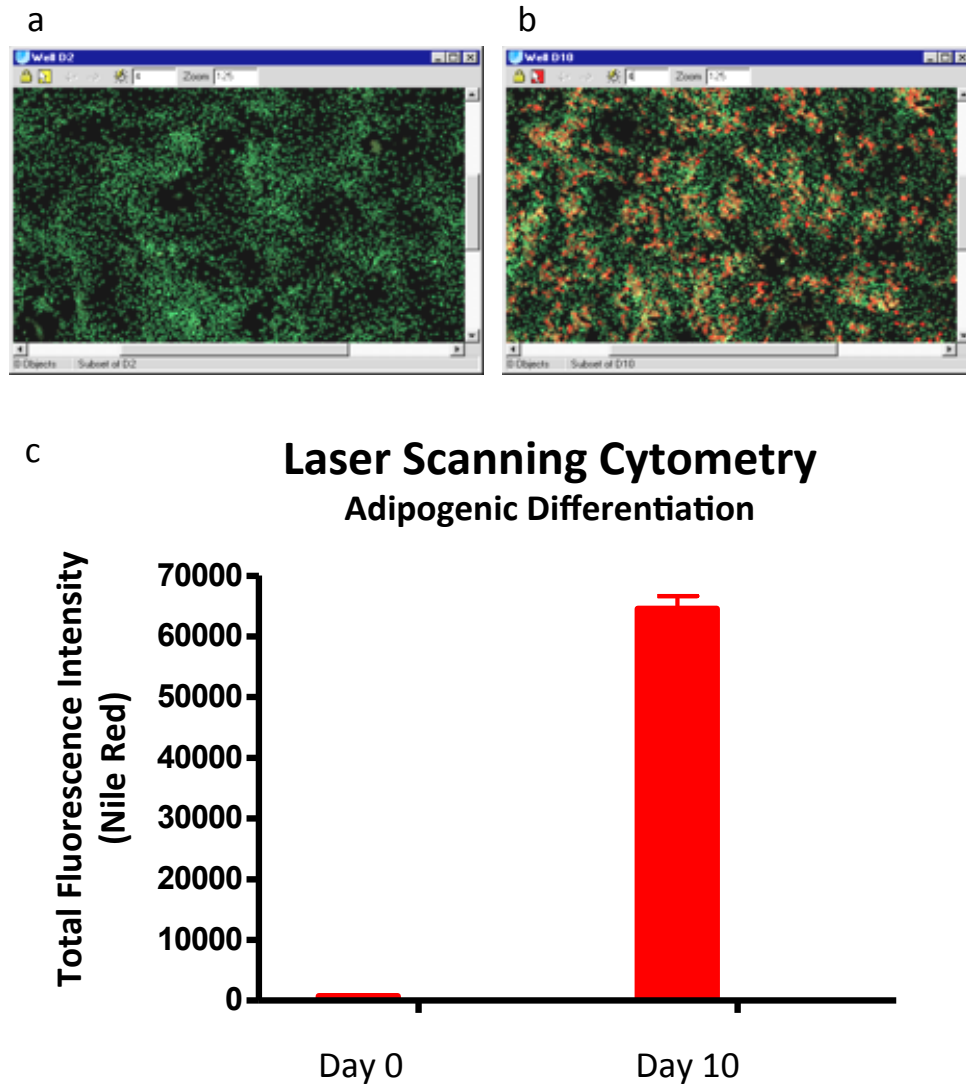
As mentioned previously, when using multiple donors of primary cells for differentiation it is critical that extent of differentiation and lineage commitment be quantified and not simply observed. Adipogenic differentiation is often identified by the presence of lipid droplets in the cytosol of cells. This is typically visually inspected using Oil Red O. However, this is non-quantitative. We developed a novel quantitative measurement of lipid accumulation using an automated fluorescence high content imaging approach leveraging the emerging field of laser scanning cytometry.

Fluorescence staining of lipids is achievable using a number of fluors, we used Nile Red for our studies in these applications. Our initial approach was to use this stain in combination with Syto16 (green nuclear stain) (Fig. 5a, b). Fixing cells at various time points during differentiation with subsequent staining demonstrated an observable increase in lipid accumulation over time (Figure 7). By imaging entire populations with laser scanning cytometry (Fig. 8a, b) and subsequent quantitation of total lipid fluorescence, lipid accumulation could be quantified (Fig. 8c). Applying this laser scanning cytometry technique with inclusion of nuclear identification and quantitation for normalization to the time course of differentiation demonstrated data that showed a biphasic curve with an increase in lipid accumulation up to day 7 followed by a decrease in lipid accumulation at later timepoints (figure 9). The visual observation and imaging data did not agree.



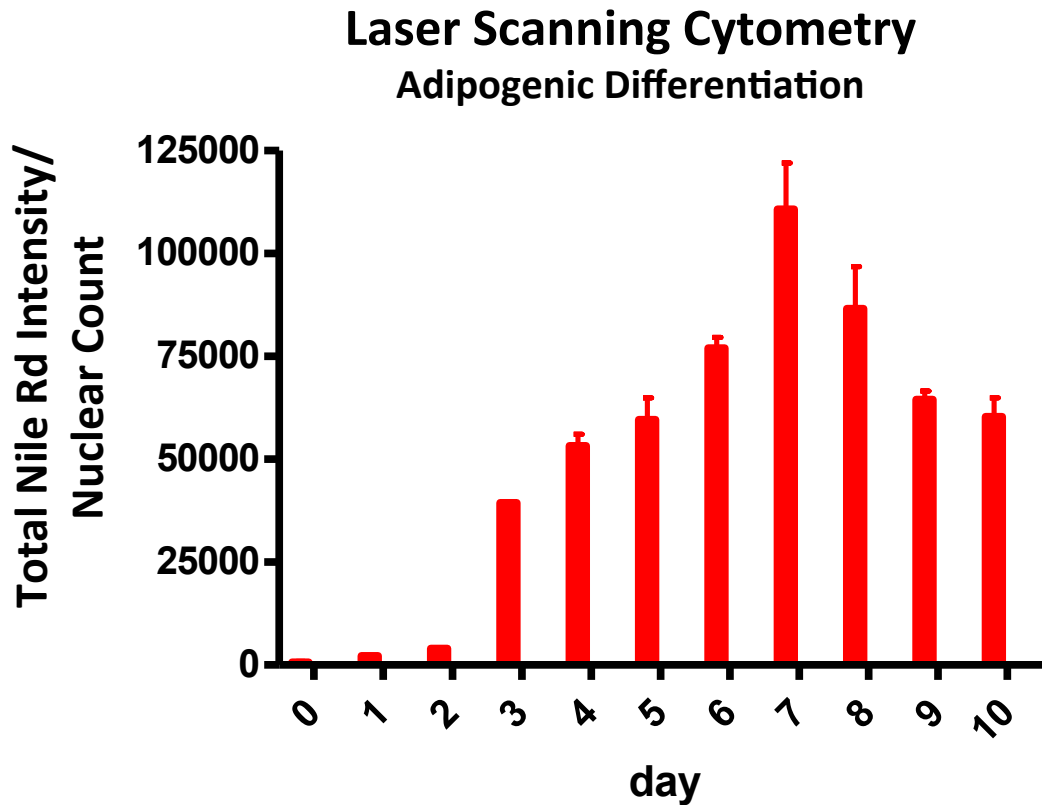
**Figure 7. Timecourse of adipogenic differentiation.** Expanded ASCs were differentiated over a 10 day period. Wells were fixed each day and stained for lipid accumulation as described in Fig 5 then imaged using fluorescence microscopy.





**Figure 8. Quantitative laser scanning cytometry of undifferentiated and differentiated adipocytes.** Undifferentiated and differentiated cells were imaged and subsequently analyzed for lipid accumulation using laser scanning cytometry (LCM) as described in methods. Image capture of undifferentiated (a) differentiated (b) ASCs, and subsequent analysis of Nile Red fluorescence (c).





**Figure 9. Laser scanning cytometry does not agree with visual inspection of lipid accumulation.** ASCs were differentiated and fixed daily over timecourse followed by staining as described in Fig 7. Fluorescence intensity from Nile Red was normalized against nuclear count.

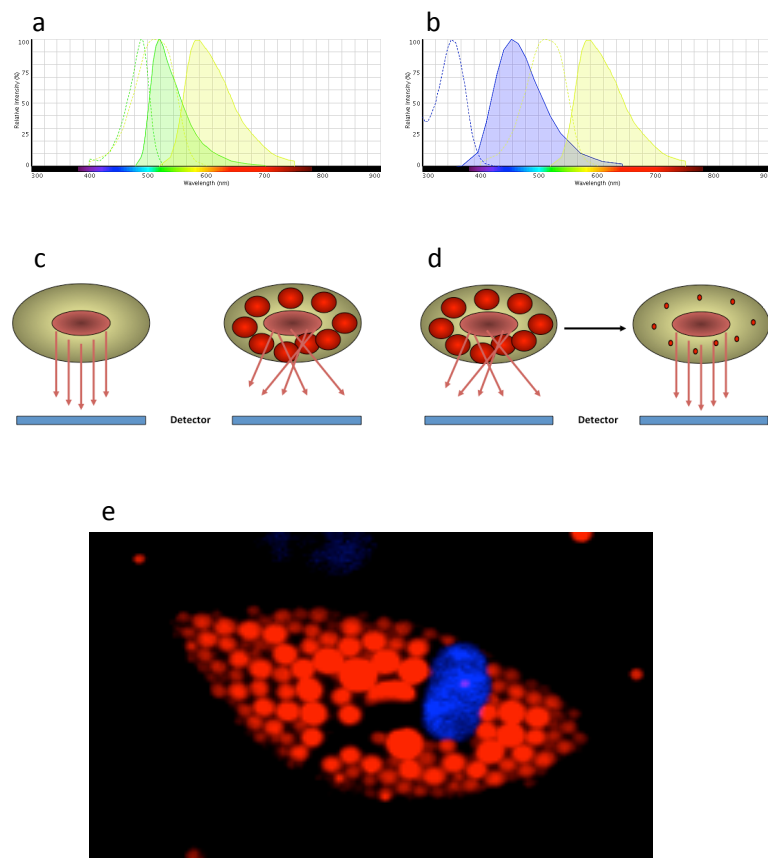
Further evaluation of our initial protocol demonstrated two potential reasons for the disconnect between visual observation and imaging data: spectral overlap between fluors used and light scattering from lipid accumulation in differentiated adipocytes. A high degree of spectral overlap between Nile Red and Syto16 lead to skewed fluorescence intensity data (Fig. 10a). To overcome this we switched to the use of

Hoechst 33342 which has a very little spectral overlap with Nile Red and perhaps more importantly required excitation using a different laser line (Fig. 10b). This separate excitation allowed separate capture of nuclear fluorescence (Hoechst) and lipid fluorescence (Nile Red). The high degree of lipid in the cells posed problems with imaging clear nuclei due the refractive index of lipid in the cytosol scattering fluorescent light from nuclei (Fig. 10c). To improve nuclear imaging and quantitation we stained and imaged lipid and nuclei in sequence with a lipid extraction in between. The cells were initially stained for lipid with Nile Red and scanned to quantify lipid accumulation, then lipid was extracted from the samples with methanol. With cytosolic lipid reduced, the nuclei were then stained with Hoechst and re-imaged to determine nuclear fluorescence (Fig. 10d). The resulting staining generated an image of adipocytes consisting of red lipid droplets and blue nuclear fluorescence (Fig. 10e). Differentiation along a timecourse and analyzed using this refined staining method demonstrated lipid accumulation in ASCs placed under adipogenic conditions and compared to ASCs prior to differentiation (Fig. 11a, b). ASCs and control population HDF cells placed under differentiation, fixed in an undifferentiated state, at 6 days and 12 days post induction demonstrated different morphologies when stained using this new method (Fig. 11c). This indicated the cells uniquely differentiate when placed under differentiation conditions when compared against a fibroblast cell line having similar cell surface marker characteristics (CD31<sup>-</sup>, CD45<sup>-</sup>, CD140b<sup>+</sup>) (Fig. 4a-d).

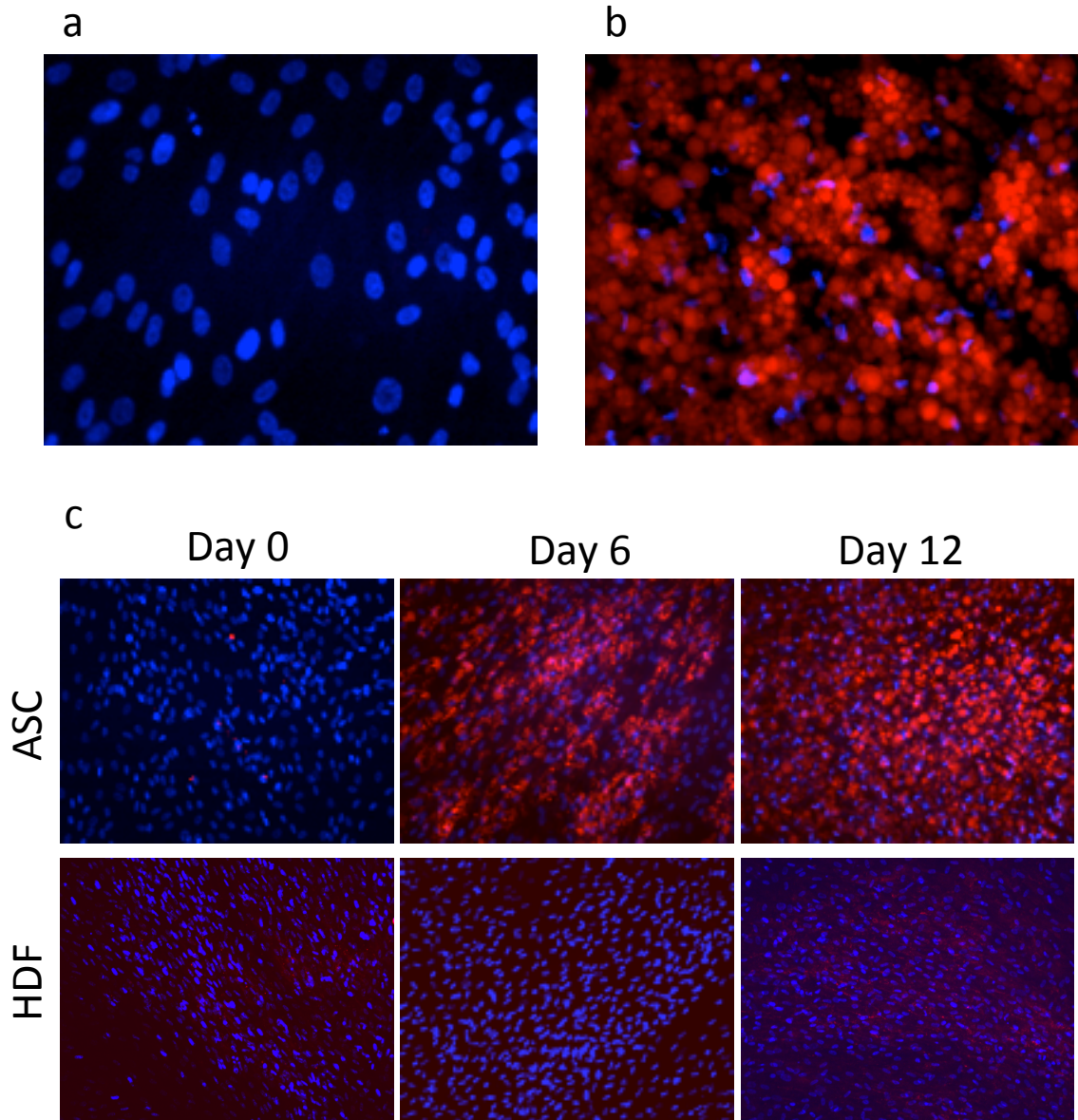
Furthermore, we found that using laser scanning cytometry we could set a threshold for lipid droplet fluorescence (using peak intensity) that allowed us to collect

lipid fluorescence only from the droplets and not background fluorescence in the samples (Fig. 12a-f). Normalizing lipid fluorescence accumulation against nuclear fluorescence using this revised staining technique and laser scanning cytometry analysis generated data that agreed with visual inspection over a timecourse of differentiation using ASCs and HDF (negative control) (Fig 12g).

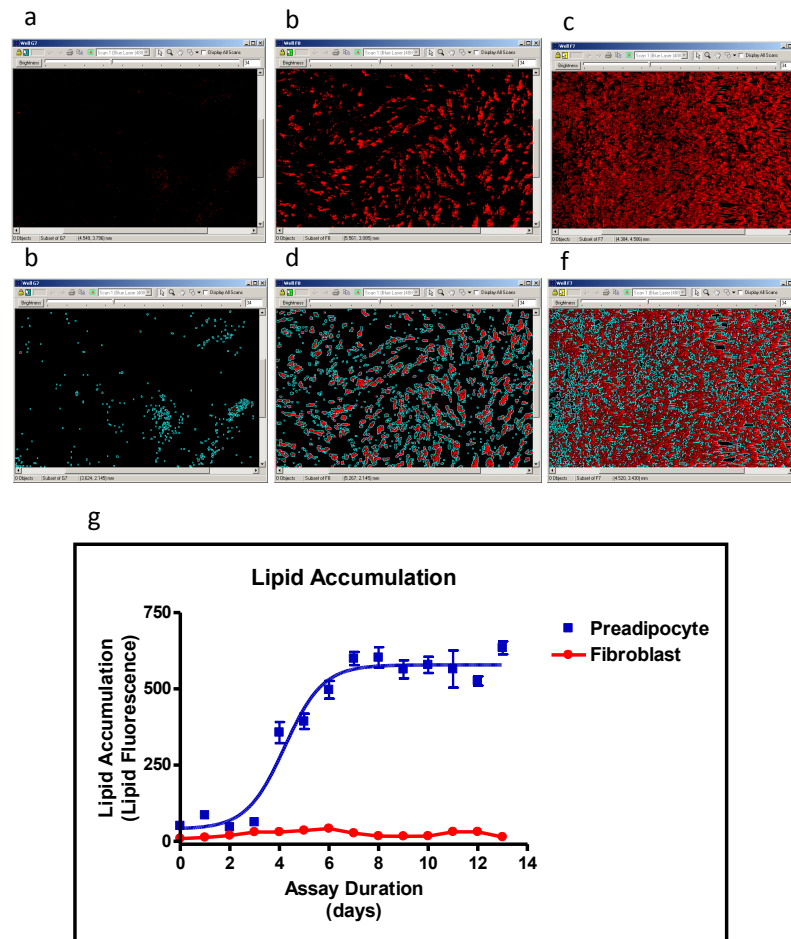
This method provides a quantitative method for lipid accumulation far superior to visual inspection of Red Red O staining. This could be used to determine extent to which differentiation could be achieved with various donors and whether or not various treatments during differentiation would affect the progression of differentiation.



**Figure 10. Alternative staining and sample preparation approach to quantifying lipid accumulation.** Excitation/emission spectra of Syto 16, green, and Nile Red, yellow (a). Hoechst, blue, substituted in for Syto16 provides better spectral separation from Nile Red and allows for a separate excitation source (b). Schematic of light scatter by lipid droplets in differentiated cells as compared to undifferentiated cells making nuclear fluorescence difficult to quantify in differentiated cells (c). Schematic of proposed solution involving lipid extraction to reduce nuclear light diffraction (d). Image of a single differentiated adipocyte Hoechst/Nile Red and two stage image acquisition and overlay, lipid image acquisition followed by lipid extraction and nuclear image acquisition (e).



**Figure 11. Imaging lipid accumulation using modified technique over adipogenic timecourse in ASCs and HDF.** Undifferentiated (a) and differentiated (b) ASCs using modified staining approach Described in Methods and Figure 10. ASCs differentiated and fixed over multiple timepoint compared to a similarly treated non-differentiating control cell line HDF (c).



**Figure 12. Thresholding using laser scanning cytometry accurately analyzes lipid accumulation by fluorescence.** Thresholding on object peak fluorescence intensity during laser scanning cytometry allows identification of fluorescence from lipid droplets and not background. Thresholding was applied in analysis algorithm for samples fixed and stained with image/lipid droplets outlined in blue at day 0 (a, d), day 4 (b, e), and day 12 (c, f) of differentiation. Using thresholding and the modified staining method described in fig 10 and Methods ASCs and non-differentiated human dermal fibroblasts were differentiated and fixed at multiple timepoints and analyzed (g).

## **A.5 Adipogenic model: depot specific/method specific studies**

Different depots of adipose tissue demonstrate various physiologies in humans. Most human adipocyte differentiation studies use primary adipocytes or differentiated preadipocytes from subcutaneous depots. However, as phenotype and contribution to metabolic syndrome are different between different adipose depots [497-499], we felt that if differentiation of preadipocytes from omental depots was feasible, it would be interesting to look at metabolic phenotypes in differentiated omental adipocytes as well as subcutaneous adipocytes. To make inquiries about metabolic changes in differentiated adipocytes where measurements are made on whole populations, a high degree of differentiation must be achieved. Using a matched pair of ASCs (isolated from the same subject) from omental adipose tissue and subcutaneous adipose tissue we compared extent of differentiation between these two depots. We tested whether the addition of PPAR gamma agonist would aid in achieving a more complete differentiation.

As preadipocytes from omental adipose tissue historically differentiate with a different capacity compared to subcutaneous preadipocytes depending on the condition of the donor [500, 501], we tested multiple differentiation techniques employing PPAR $\gamma$  agonists in multiple formats in hopes of identifying conditions for a complete differentiation of the omental-derived cells. These included differentiation without PPAR gamma agonist, with PPAR gamma agonist for the “normal” amount of time (first half of differentiation) and leaving the PPAR $\gamma$  agonist throughout the entire

differentiation to determine whether we might find increased differentiation with increased PPAR agonism in the omental preadipocytes.

Subcutaneous ASCs differentiated extensively over time. Microscopy at various time points throughout differentiation demonstrated similar morphometric changes between the two groups when PPAR gamma agonism was included in their differentiation procedure. They both appear to dramatically increase lipid vesicle accumulation between days three and six and appear to have similar fractions (near 100%) of the total population differentiated by the end of the time course (Fig. 13a). In contrast, differentiation without PPAR gamma agonist demonstrated a delayed increase in lipid accumulation in the subcutaneous cells on days nine through twelve and a smaller fraction of the total population differentiated by the end of the time course (Fig. 13a).

In comparison, the omental ASCs from the same donor differentiated using the same conditions demonstrated less overall differentiation in comparison to the subcutaneous ASCs. Differentiation with PPAR gamma agonist (both limited to the first half and throughout differentiation) demonstrated increased appearance of lipid droplets over time with the majority of increase seen between days nine and twelve (Fig. 13b). Whereas the subcutaneous cells appeared to have similar amount of differentiation by day 12 regardless of PPAR gamma agonist duration, the omental cells appear to differentiate to a fuller extent (larger portion of the total population) with the use of full term PPAR gamma agonism (Fig. 13b). Differentiation of omental ASCs



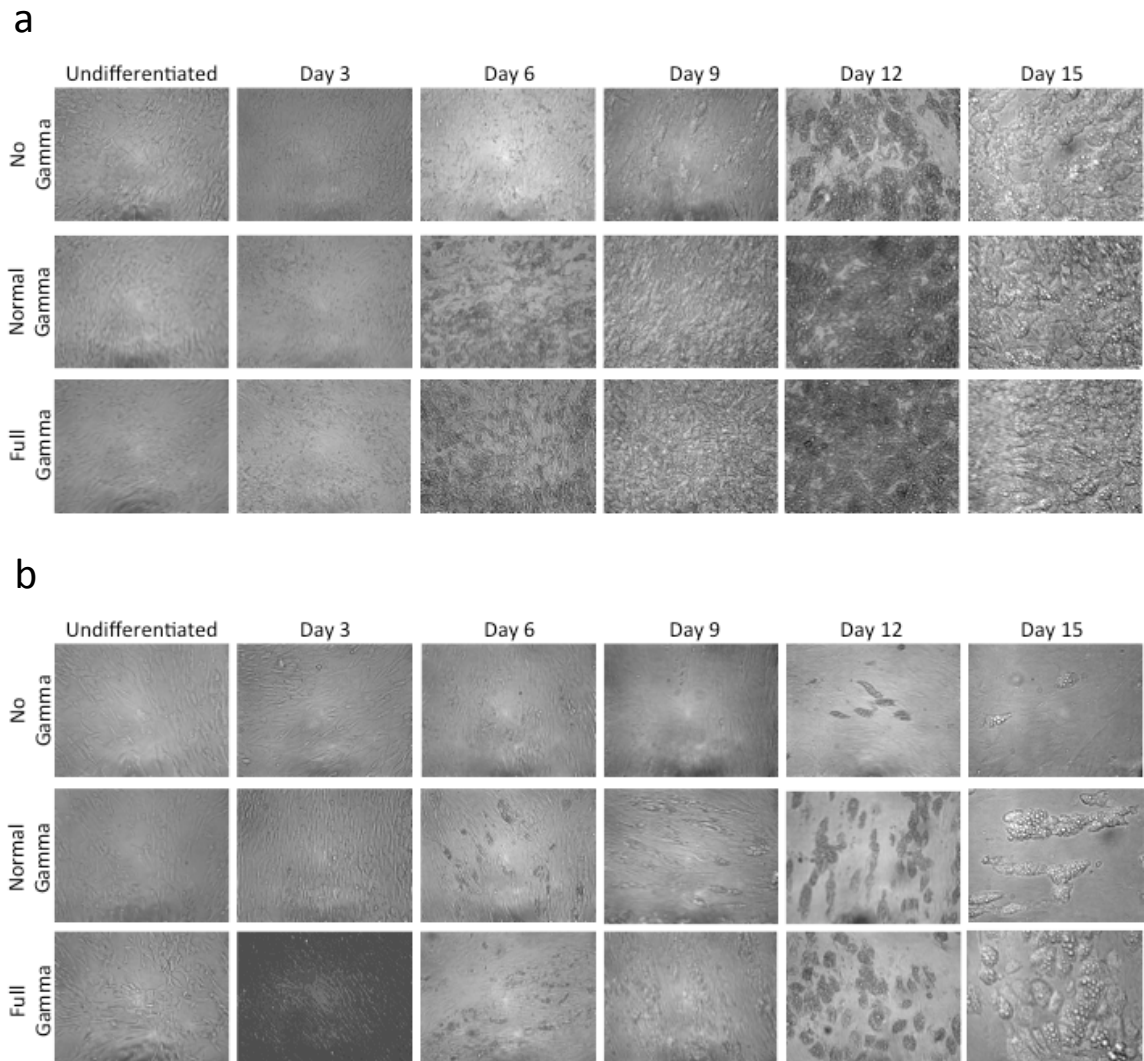
without addition of PPAR gamma agonist resulted in little to no differentiation into mature adipocytes (Fig. 13b).

Similarly, when differentiated in parallel and fluorescently stained for lipid accumulation using previously described method, increased lipid accumulation is observed in subcutaneous preadipocytes differentiated with some PPAR gamma agonist present (Fig. 14c, d). Subcutaneous ASCs differentiated without addition of PPAR gamma showed decreased lipid staining (Fig. 14b) compared to differentiation with PPAR gamma agonist. Cells maintained throughout the time course with no differentiation induction at all demonstrated little to no lipid staining (Fig. 14a). Omental cells showed little to no lipid staining in cells maintained throughout time course without differentiation induction and with differentiation induced in the absence of PPAR gamma agonist (Fig. 14e, f). Minimal lipid staining was observed in cells differentiated with PPAR gamma agonist added traditionally (Fig. 14g) and an increased amount of lipid staining was observed in omental ASCs differentiated with PPAR gamma agonist addition through the duration of differentiation (Fig. 14h).

Quantitation of staining over time course using the above-described laser scanning cytometry method validated both morphometric and fluorescence staining. Subcutaneous ASCs differentiate to a fuller extent (by lipid accumulation measurement) with some PPAR gamma agonism (Fig. 15a). Whether PPAR gamma agonist is added throughout differentiation or more traditionally during the first half of differentiation, a similar amount of lipid accumulation is quantified by the end of differentiation. Without

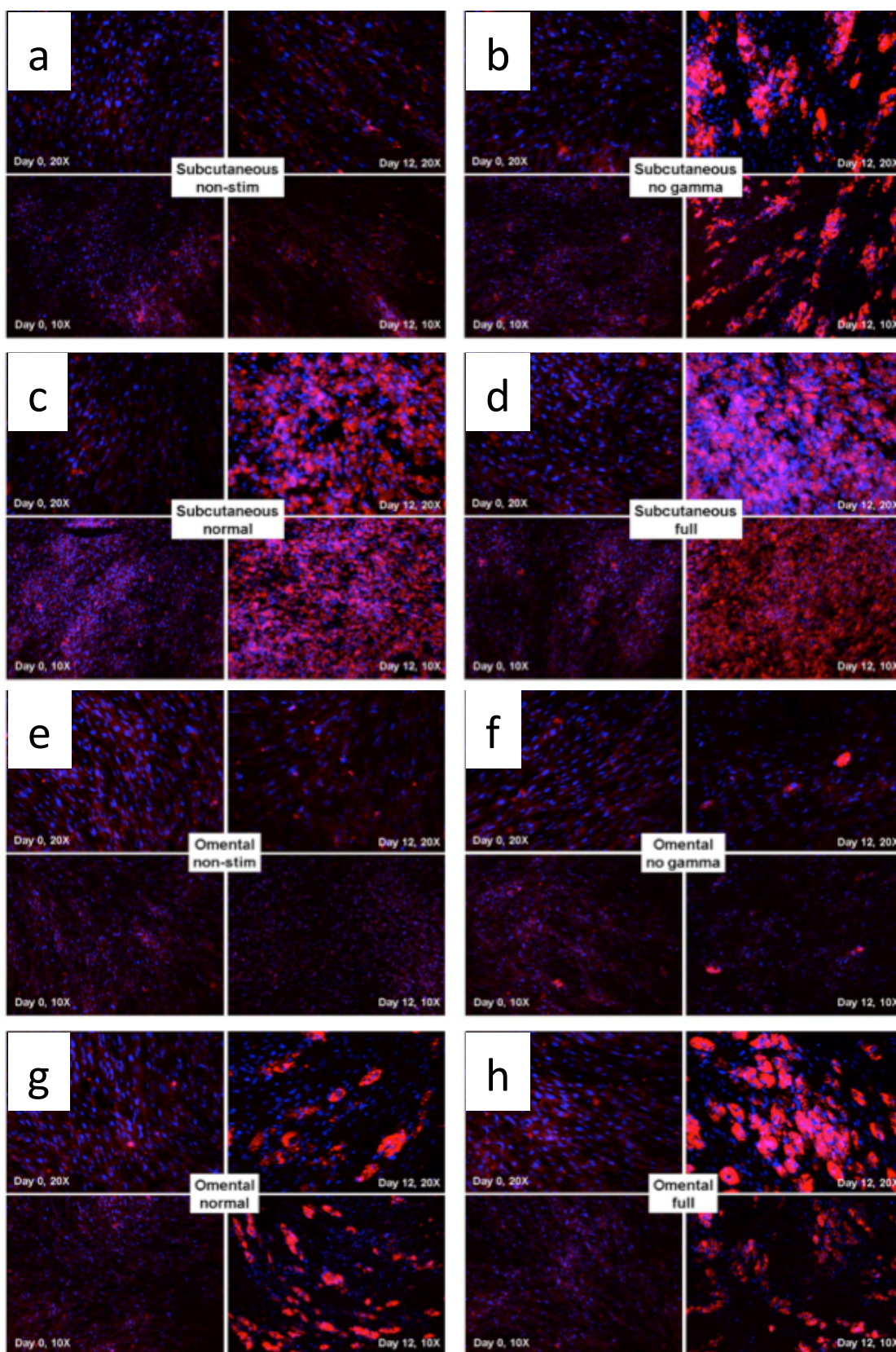
PPAR gamma agonism, significantly less differentiation is achieved and lipid accumulation appears to occur primarily at later timepoints than differentiation with PPAR gamma agonist included (Figure 15a). Omental ASCs differentiated to a much lesser extent and achieved increased lipid accumulation only when differentiation included addition of PPAR gamma agonist. A stark increase in lipid accumulation was observed with PPAR gamma agonist addition for the duration of differentiation as compared to addition only during the first half of differentiation (Fig. 15b).

Comparison of differentiation using omental and subcutaneous ASCs demonstrated a substantially more complete differentiation using subcutaneous ASCs. Omental cells demonstrated a low degree of differentiation that would not lend itself to metabolism studies in differentiated adipocytes as measurements post differentiation would be on a mixed population of adipocytes and undifferentiated cells whether differentiation is stimulated with PPAR gamma agonist or not. The subcutaneous cells however produce a very homogeneous population post differentiation with a high degree of differentiation when PPAR gamma agonist is included during differentiation. These findings however demonstrate that PPAR gamma agonist does not need to be used throughout the entire differentiation as similar results are achieved using PPAR gamma agonism in the beginning of differentiation. While omental adipose is an interesting adipose depot, we decided to focus my research on the metabolic phenotype of differentiated adipocytes using subcutaneous ASCs, given the extent to which differentiation is achieved in a vast majority of the population whereas only a small portion of the omental cells differentiated.

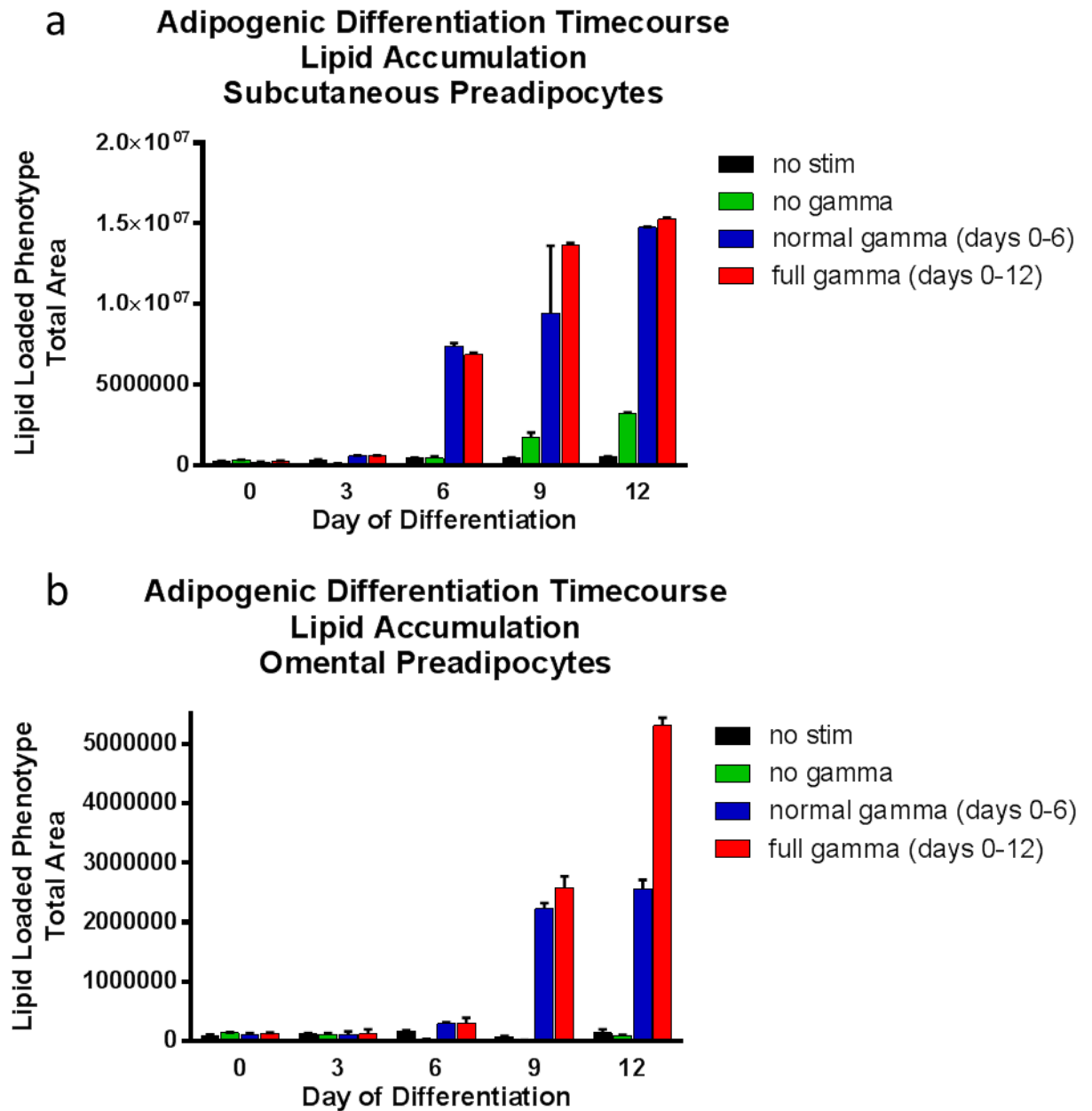


**Figure 13. Differentiation of ASCs from multiple depots with multiple conditions.**

ASCs from both subcutaneous (a) and omental (b) fat isolated as a matched pair from a single donor were differentiated under varying conditions of PPAR gamma stimulation: no PPAR gamma agonist, “normal” PPAR gamma agonist time course (first half of differentiation), and PPAR gamma agonism throughout differentiation. Cells were fixed and imaged at various timepoints throughout differentiation.



**Figure 14. Fluorescence staining of differentiating ASCs from multiple depots/multiple conditions.** Multiple depots/multiple methods of differentiation were tested as described in Figure 13. Cells were fixed prior to differentiation and after 12 days of differentiation, stained and imaged as described in Fig 5. Multiple magnifications are shown of subcutaneous and omental (a, e) ASCs with no differentiation stimulation; differentiation with no gamma agonist (b, f); differentiation with typical PPAR gamma agonist added in the first half of differentiation (c, g); and differentiation with PPAR gamma agonist exposure throughout (d, h).



**Figure 15. Lipid accumulation in differentiating ASCs from multiple depots/multiple conditions over timecourse.** Lipid accumulation quantitation using previously described laser scanning cytometry method (Fig 12) comparing subcutaneous(a) and omental (b) derived ASCs differentiated as described in Figures 14 at multiple timepoints.

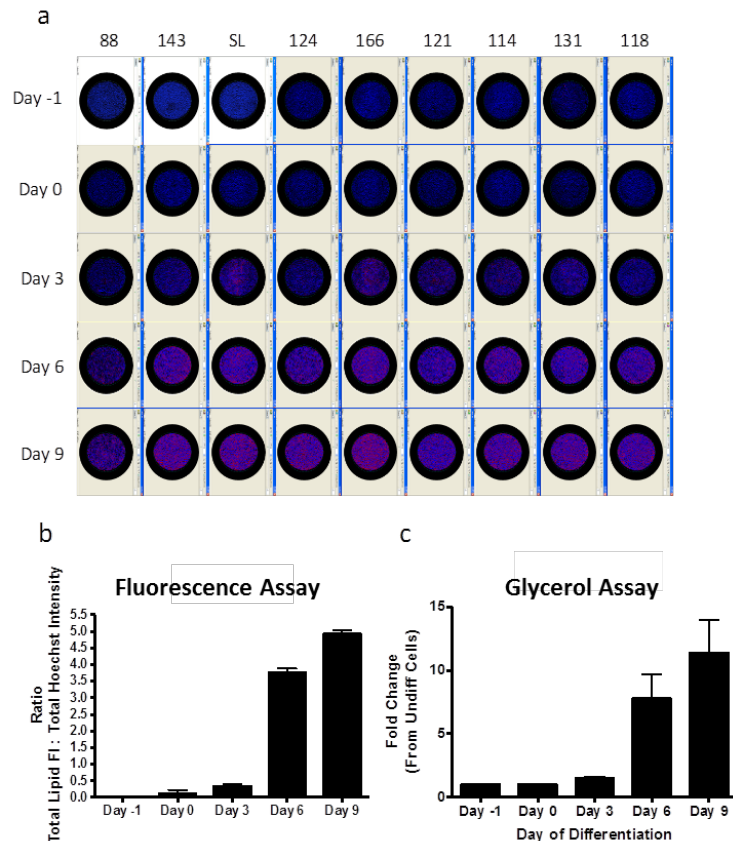


## **A.6 Consistency across donors**

Further metabolism studies in differentiated adipocytes were performed using differentiated ASCs from multiple donors to rule out single donor primary culture phenomena. To determine donor-to-donor consistency of differentiation, multiple donors were placed under adipogenic differentiation conditions. Extent of differentiation was determined using the quantitative lipid accumulation method previously described along with glycerol analysis as an additional measure of lipid accumulation as a means of validating our novel method of measuring lipid accumulation by automated imaging/laser scanning cytometry.

Similar levels of lipid accumulation were observed across multiple donors at multiple timepoints with similar distribution of differentiated (lipid laden) cells across the populations when observing images capture by laser scanning cytometry (Fig. 16a). Quantitation of lipid accumulation using the previously described method demonstrated that ASCs from all donors tested (including a “superlot” mix of all donors) demonstrated similar differentiation over time. All showed an increase in lipid accumulation primarily between days three and six and achieved similar levels of lipid staining by the end of the time course (Fig. 16b). To verify these findings, de-esterified glycerol (fatty acids removed) was assessed as a surrogate of triacylglycerol content in the differentiating populations. Glycerol data was similar to lipid staining approach demonstrating the largest accumulation of lipid between days three and six of differentiation and similar increases in glycerol were achieved among all donors tested (Fig. 16c).

These data taken together demonstrate similar levels of differentiation amongst all donors tested. This is critical as these decrease the probability that data trends could be from variable levels of differentiation when using multiple donors.

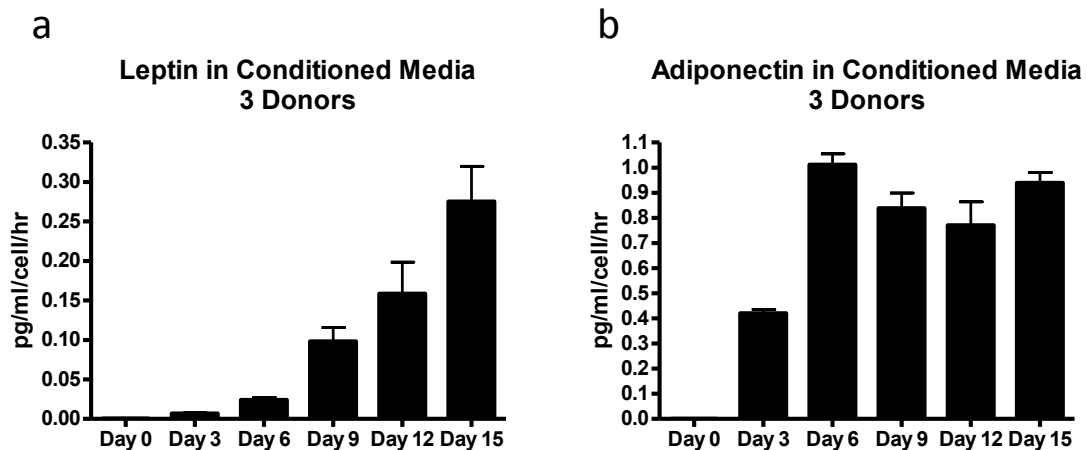


**Figure 16. Comparison of adipogenic differentiation using subcutaneous ASCs from multiple donors.** ASCs isolated from eight donors and a combined superlot (SL) were differentiated as described in Methods and subsequently analyzed for differentiation using method described in Figure 12. Representative images are shown of each of the donors tested over a 9 day period (a). Quantitative laser scanning cytometry was performed on samples (b). In parallel glycerol accumulation was measured as described in methods in the same populations (c).



## **A.7 Adipokine secretion**

Differentiation state of ASCs down the adipogenic lineage is most commonly characterized by lipid accumulation as we have already demonstrated. However, a secondary approach to better characterize differentiation down the adipogenic lineage is necessary. Multiple cell types (hepatocytes, macrophage, etc.) can lipid load, especially in culture making a secondary non-lipid measurement valuable in demonstrating true adipogenic lineage differentiation. To test this in our model we examined secretion of adipokines into media at multiple times throughout differentiation. We profiled both adiponectin and leptin secretion via ELISA on conditioned media which are only secreted by adipocytes. Multiple donors were tested at days 0, 3, 6, 9, 12, and 15 of differentiation. With adipogenic differentiation we expect to see an increase in secretion of these adipokines over time as compared to baseline (day 0). Leptin secretion increased as the cells differentiated (Fig. 17a). Similarly adiponectin increased as the cells differentiated (Fig. 17b) with an initial increase much earlier than leptin. The data are another indicator the cells are differentiating down the adipogenic lineage.



**Figure 17. Adipogenic differentiation of multiple donors demonstrates increased production of both leptin and adiponectin.** ASCs isolated from three donors were analyzed for adipokine production in conditioned media at multiple times throughout adipogenic differentiation by ELISA. Both leptin (a) and adiponectin (b) production were measured.

#### A.8 Data summary

My data indicate that using human ASCs, one can model adipogenic differentiation with a high degree of reproducibility across multiple donors. A novel imaging assay was developed enabling high content imaging of entire populations of cells to determine lipid accumulation across whole populations quantitatively as opposed to standard oil red o staining. Omental ASCs demonstrated a much reduced capacity for adipogenic differentiation than did subcutaneous ASCs under the conditions tested. Both adiponectin and leptin secretion increase with differentiation with

adiponectin increases and peaking at earlier timepoints. The consistency across donors and high degree of differentiation compare favorably with 3T3-L1 models which are valued for their reproducibility as a cell line and indicate that differentiation studies using multiple donors of ASCs are indeed feasible and not limited by variability.

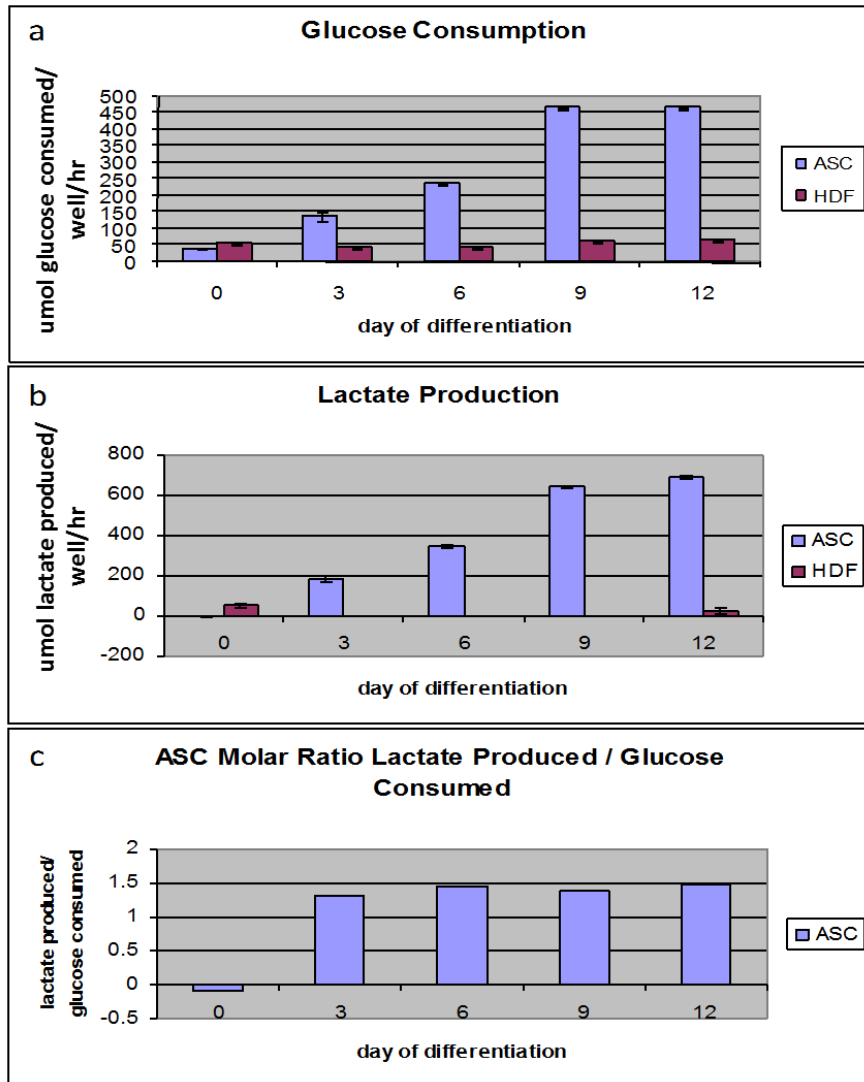
## **B. Aerobic Glycolysis in Differentiated Adipocytes**

To test whether differentiated adipocytes exhibit the ability to perform aerobic glycolysis, glycolytic and oxidative measurements were performed using differentiated adipocytes compared to their undifferentiated precursors (ASCs). The undifferentiated adipocyte is considerably different from the mature adipocyte yet is from the same source material and therefore functions as the best control available. This also allows us to make inferences as to metabolic changes that are associated with the changing role of the mature adipocyte compared to its precursor.

### **B.1 Preliminary findings**

Using our model of differentiation, conditioned media was captured throughout differentiation and analyzed using a clinical blood analyzer for lactate and glucose concentrations to look at both glucose consumption and lactate production by comparing to unconditioned media controls. Glucose consumption increased with differentiation (Fig. 18a). The mature cell has a primary role of energy storage so increasing flux of high energy molecules such as glucose is expected. However, at the

same time, lactate production also increased (Fig. 18b). When comparing the molar ratio of lactate produced to glucose consumed, the data indicated that the cells in an undifferentiated state had a low ratio (little lactate produced per glucose consumed). However, as the cells differentiated they increased this ratio (higher amounts of lactate produced per glucose consumed) (Fig. 18b). Fibroblasts put under the same differentiation conditions as a control (non-differentiating) did not exhibit any of these findings (Fig. 18c). This became the preliminary data that led to the hypothesis that during differentiation, and in the differentiated state, adipocytes exhibit an increase glycolytic rate of metabolism relative to the oxidative rate of metabolism.

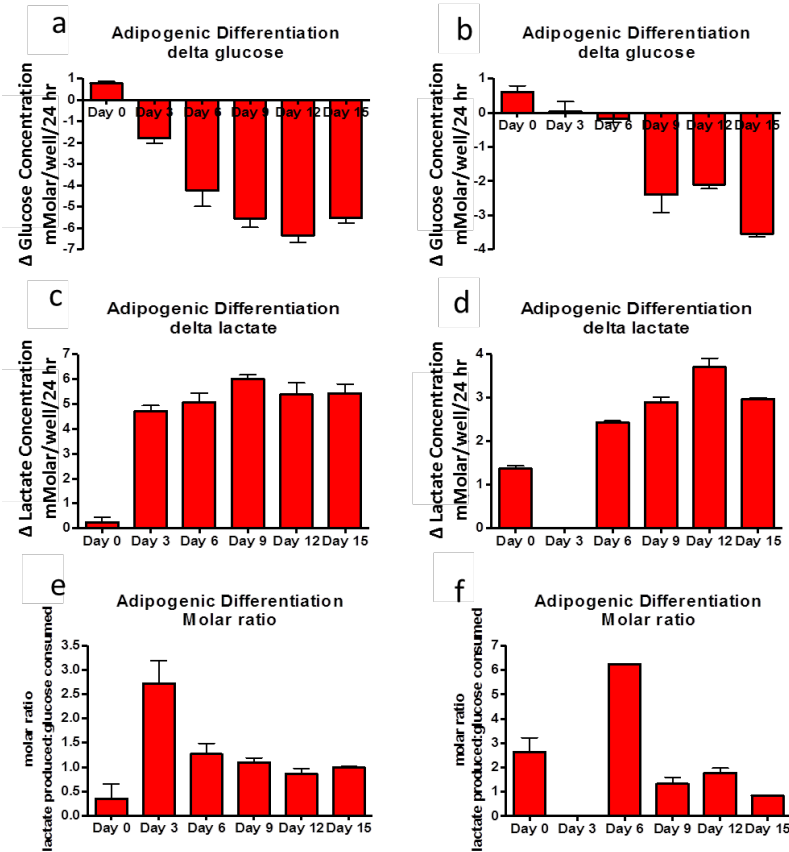


**Figure 18. Glucose consumption and lactate production disproportionately increase with adipogenic differentiation.** Conditioned media from differentiating ASCs and HDF were collected at various timepoints and analyzed using a clinical blood analyzer to determine glucose consumption (a), lactate production (b). This data was used to determine lactate produced per glucose consumed (c), only for the differentiating adipocyte.

## **B.2 Adipose depot specific metabolism**

As previously mentioned, poor amounts of adipogenic differentiation are achieved using omental preadipocytes. However, with a matched pair (omental and subcutaneous preadipocytes) from a single donor, we compared glucose consumption and lactate production rates during differentiation using precursors from both depots. Glucose consumption and lactate production accompanying differentiation in both omental and subcutaneous models revealed similar results. An increase in glucose consumption in both omental and subcutaneous models (Fig. 19a, b) accompanied adipogenic differentiation. Glucose consumption was higher in the differentiating subcutaneous adipocytes compared to omental. Similarly, lactate production increased in both the differentiating subcutaneous and omental adipocytes (Fig. 19c, d). However, lactate production was higher in the subcutaneous adipocytes which could be from the increased amount of adipogenic differentiation in subcutaneous ASCs as compared to the omental ASCs. The molar ratio of lactate produced to glucose consumed demonstrated increases similar to preliminary data for differentiation of subcutaneous adipocytes (Fig. 19e). The ratio went above 2 in some points. This would indicate that lactate production was potentially coming from substrate other than the glucose consumed. Indeed, upon consideration of the media we used (containing L-glutamine and pyruvate) there were alternative carbon sources available for lactate production other than glucose, this made ratios of lactate produced to glucose consumed difficult to definite conclusions from. Accordingly, while this data was valuable for preliminary findings, subsequent studies were performed with a more highly controlled media.

Omental adipocyte differentiation did not demonstrate a similar increase lactate produced to glucose consumed at later timepoints (Fig. 19f).



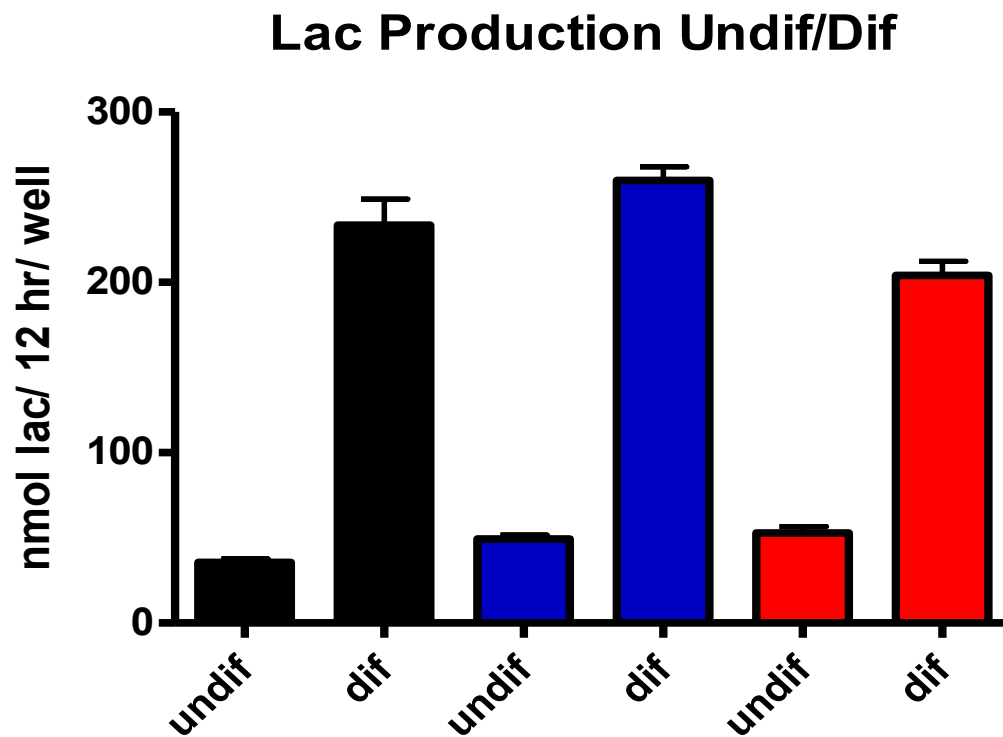
**Figure 19. Lactate production increases vs. glucose consumption in both subcutaneous and omental adipogenic differentiation.** Conditioned media from omental and subcutaneous matched donor ASCs was collected and analyzed as described in Figure 18. Increases in glucose consumption ( $-\Delta$  glucose) were observed with differentiation of both subcutaneous and omental (a, b) ASCs. Similar increases in lactate production ( $+\Delta$  lactate) were observed (c, d). An increase in ratio of lactate produced to glucose consumed was observed compared to undifferentiated or day 0 cells (e, f).

### **B.3 Lactate measurements using multiple donors in various conditions**

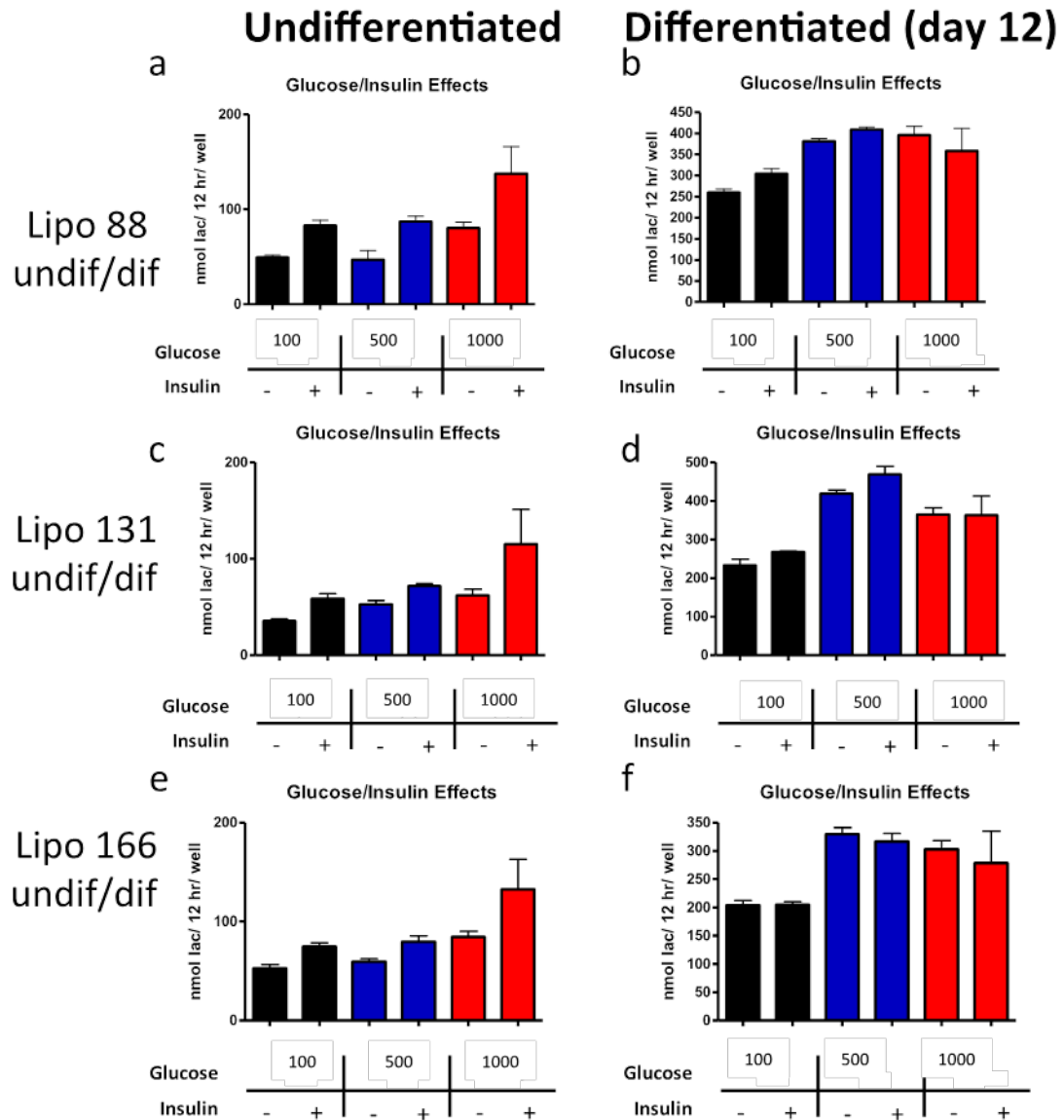
To better understand lactate production rates associated with adipocytes compared to their precursors, ASCs from several donors were obtained and differentiated using methods previously described. Lactate measurements were performed on media (controlled for glucose and devoid of glutamine and pyruvate) after conditioning by the cells in both differentiated and undifferentiated states. Each of the donors showed marked increases in lactate production after differentiation into mature adipocytes (Figure 20).

Studies up to this point had been performed euglycemic conditions (100 mg/dL glucose), Further studies were performed using multiple glucose concentrations and insulin stimulation. Insulin had little to no effect on lactate production in the differentiated adipocytes across all donors whereas increasing glucose seemed to increase lactate production at 500 and 1000 mg/dL glucose compared to euglycemic conditions at 100 mg/dL (Figure 21b, d, f). In the undifferentiated cells, insulin had a larger effect than glycemic conditions. Insulin appeared to increase lactate production (Fig. 21a, c, e). Data was consistent across all donors indicating an increase in lactate production or glycolytic metabolism accompanies differentiation. The increased lactate production with increased glucose concentration suggests that excess glucose flux into the cell above some threshold amount might be funneled into glycolytic processes. To better determine this, the lactate data would have to be compared against oxidative data under similar conditions (see later results).





**Figure 20. Lactate production is higher in differentiated adipocytes than undifferentiated ASCs in multiple donors.** Three separate donors were tested in both undifferentiated and differentiated states for lactate production by colorimetric lactate assay after media conditioning.



**Figure 21. Insulin and glucose effects on lactate production in differentiated and undifferentiated ASCs.** Lactate production was measured in conditioned media as previously described on undifferentiated (a, c, e) and differentiated ASCs(b, d, f) from three donors in various hyperglycemic conditions and with and without addition of 100 nM insulin.

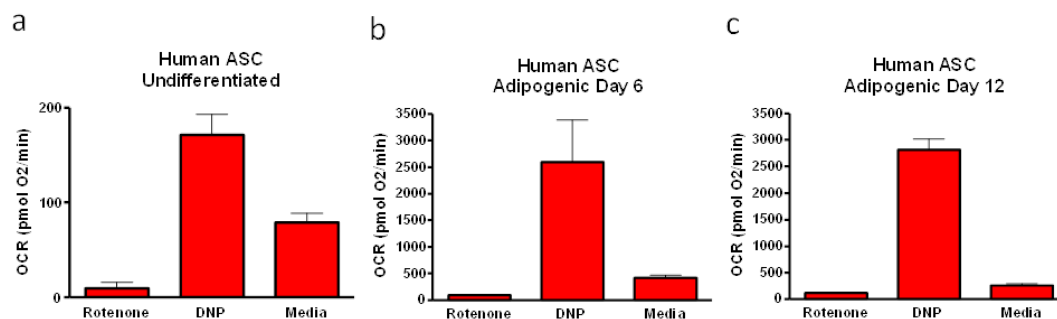
#### **B.4 Oxygen consumption measurements using multiple donors**

Oxidative measurements using Seahorse Bioscience FX analyzer allow for real-time oxygen consumption measurements by creating a low volume space around the cells and making oxygen tension measurements over time to determine oxygen consumption rates. To understand the oxidative state of the cells we employed the use of mitochondrial toxins that would allow us to measure basal oxygen consumption rates (not treatment), maximal oxygen consumption rates (uncoupling with DNP), and minimum oxygen consumption rates (inhibiting site one of electron transport with rotenone). To observe whether oxidative metabolism changes with differentiation, oxygen consumption rates were measured in undifferentiated day 0 (Fig. 22a), differentiating day 6 (Fig. 22b), and differentiated cells day 12 populations (Fig. 22c). Treatment with rotenone decreased oxygen consumption rates in undifferentiated, day 6 and day 12 adipocytes as a result of site one inhibition of electron transport chain. DNP increased oxygen consumption rates at each time point tested as a result of mitochondrial uncoupling with larger responses (increasing) observed in the day six and day 12 differentiated adipocytes compared to day 0 undifferentiated preadipocytes.

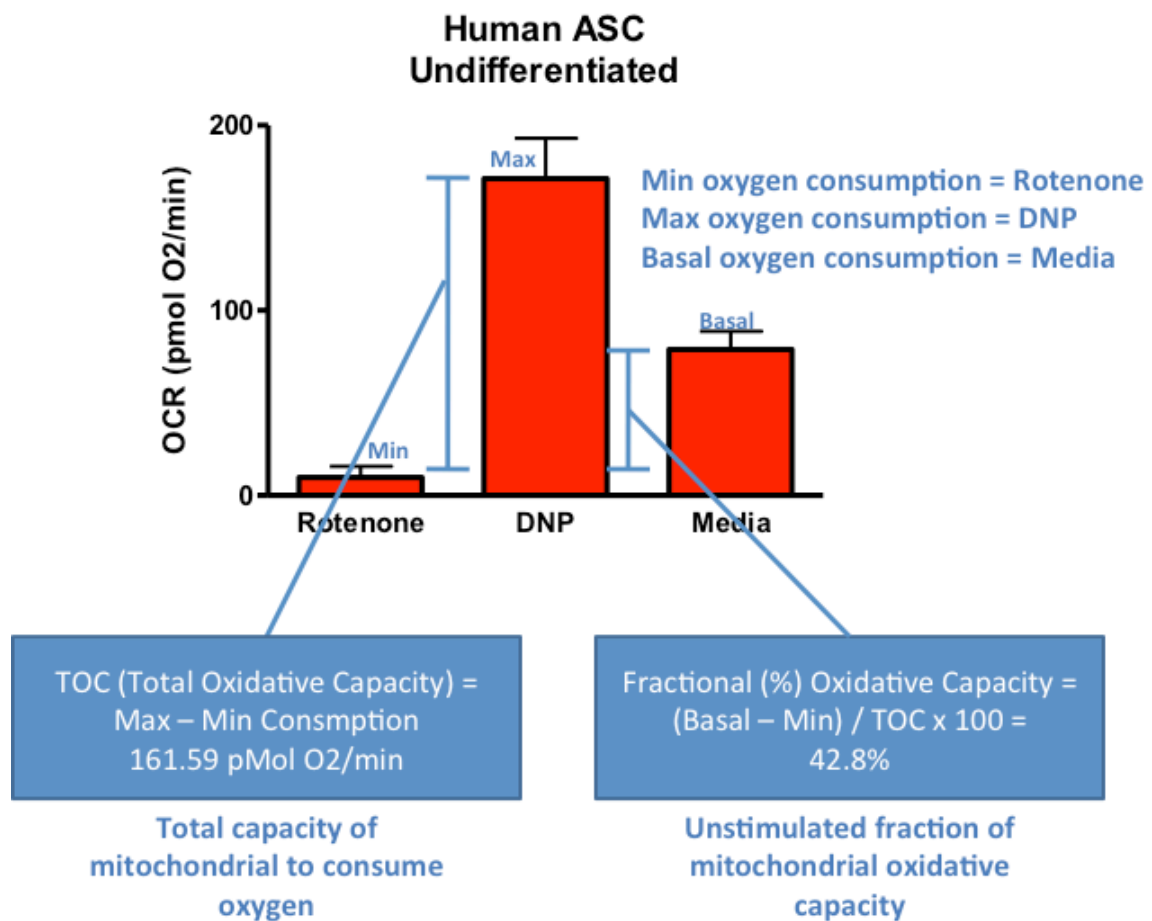
DNP treatment allows for free proton movement across the mitochondrial membrane destroying the proton gradient and the proton motive force necessary for ATP synthase activity. The cell increases oxidative phosphorylation up to maximal rates to restore this. Rotenone inhibits site one of electron transport. Using these mitochondrial toxins we are able to determine basal oxidative activity (untreated O<sub>2</sub>

consumption rate), maximal oxidative rate (DNP treated O<sub>2</sub> consumption rate), minimum oxidative rate (rotenone treated O<sub>2</sub> consumption rate). Using these measurements we could determine total oxidative capacity (max – min or DNP – Rotenone elicited O<sub>2</sub> consumption rates) (Fig 23). As a fraction of this total oxidative capacity we could observe where the cells function in a basal state (unstimulated), we termed this fractional oxidative state.

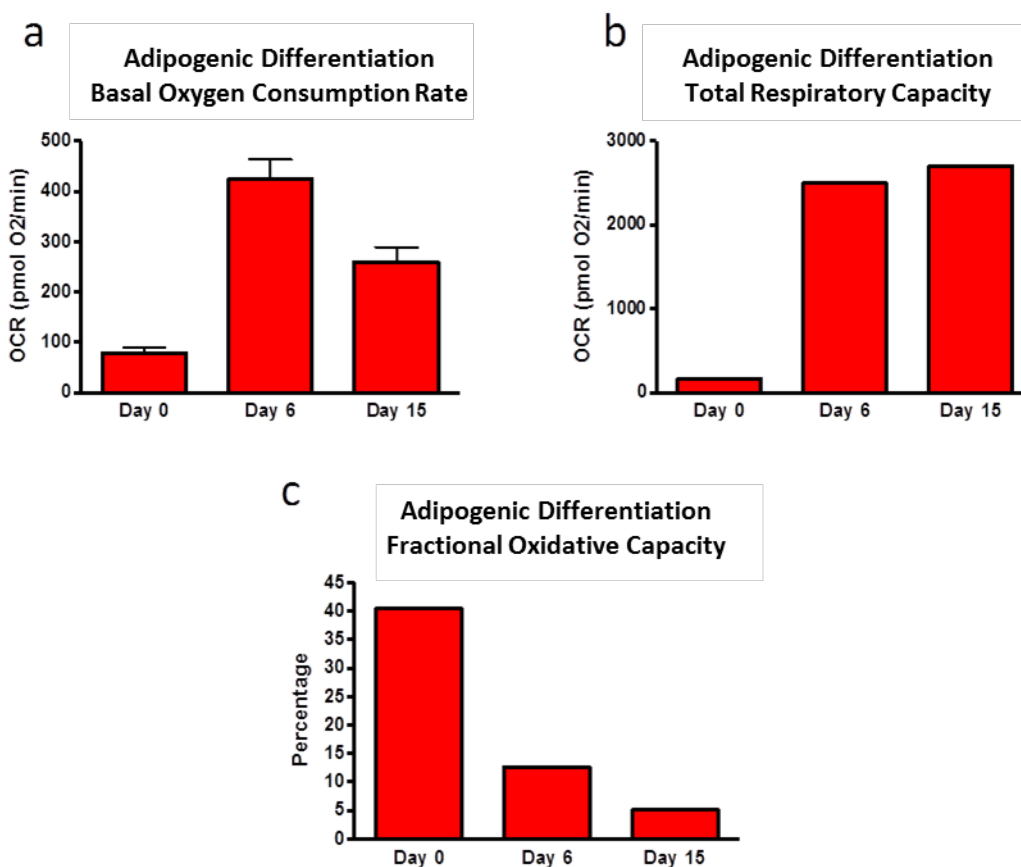
Basal oxidative rates increased with adipogenic differentiation of ASCs (Fig. 24a) indicating an increase in oxidative processes accompanying differentiation. However, total oxidative capacity also increased substantially with differentiation (Fig. 24b). As a function of this increase in total oxidative capacity the fractional oxidative state actually decreases as the cells differentiate (Fig. 24c). This indicates that the cell's oxidative process is not rate limited. One concern we had with our data demonstrating an increase in lactate production was that the increase in glycolytic activity was simply because oxidative capacity was saturated and any further metabolism would be forced through glycolytic process. This data actually shows the opposite; the differentiated adipocytes demonstrate a much larger excess capacity above basal state to perform oxidative metabolism than do the undifferentiated ASCs.



**Figure 22. Oxygen consumption in differentiating adipocytes using mitochondrial toxins.** Oxygen consumption was measured using Seahorse Bioscience XF analyzer on differentiating adipocytes. Data shown is from a single donor. Traces from Seahorse analyzer before and after rotenone, DNP, or media (control) injection from days 0, 6, 12 of differentiation (a, c, e). Corresponding values are plotted (b, d, f).



**Figure 23. Oxidative capacity and fractional oxidative capacity calculations.** Sample data demonstrating calculations for oxidative capacity and fractional oxidative capacity. Rotenone is used to inhibit mitochondrial oxygen consumption, DNP maximizes consumption, and media indicates basal consumption rates.



**Figure 24. Changes in basal, capacity, and fractional oxidative values in differentiating adipocytes.** Oxidative data in a single donor of differentiating adipocytes was obtained as described in Fig 22, 23. Basal oxygen consumption (a), total oxidative capacity (b), and fractional oxidative capacity (c) changes associated with adipogenic differentiation are shown.

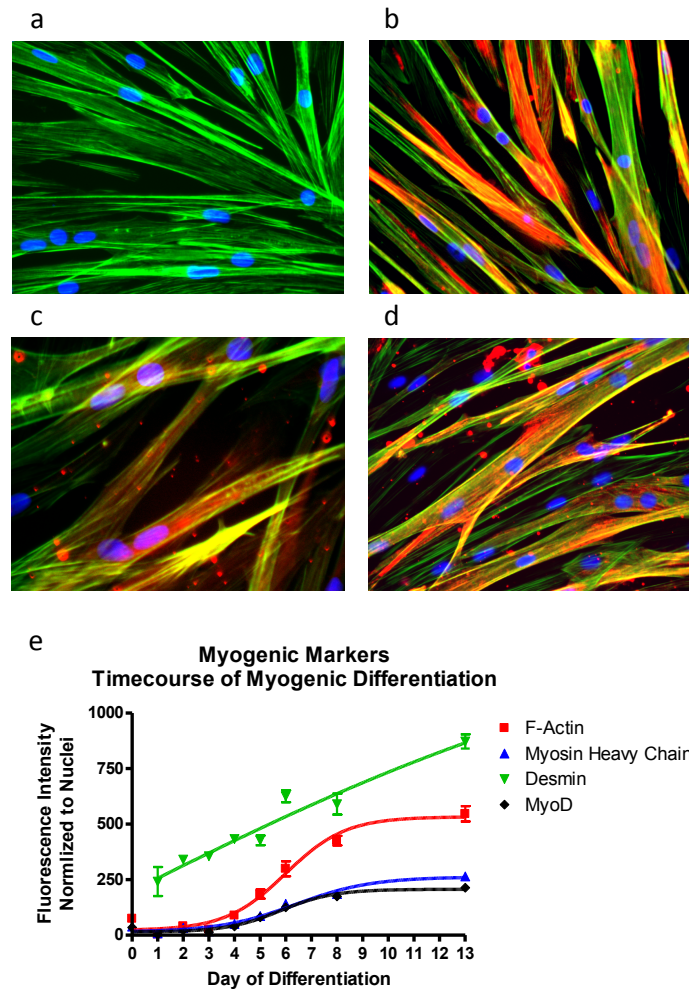
### B.5 Comparison against another differentiation model

To determine whether this observed increase in capacity and decrease in fractional oxidative state is primarily a function of differentiation *in vitro*, or was a particular characteristic of differentiated adipocytes, similar experiments were

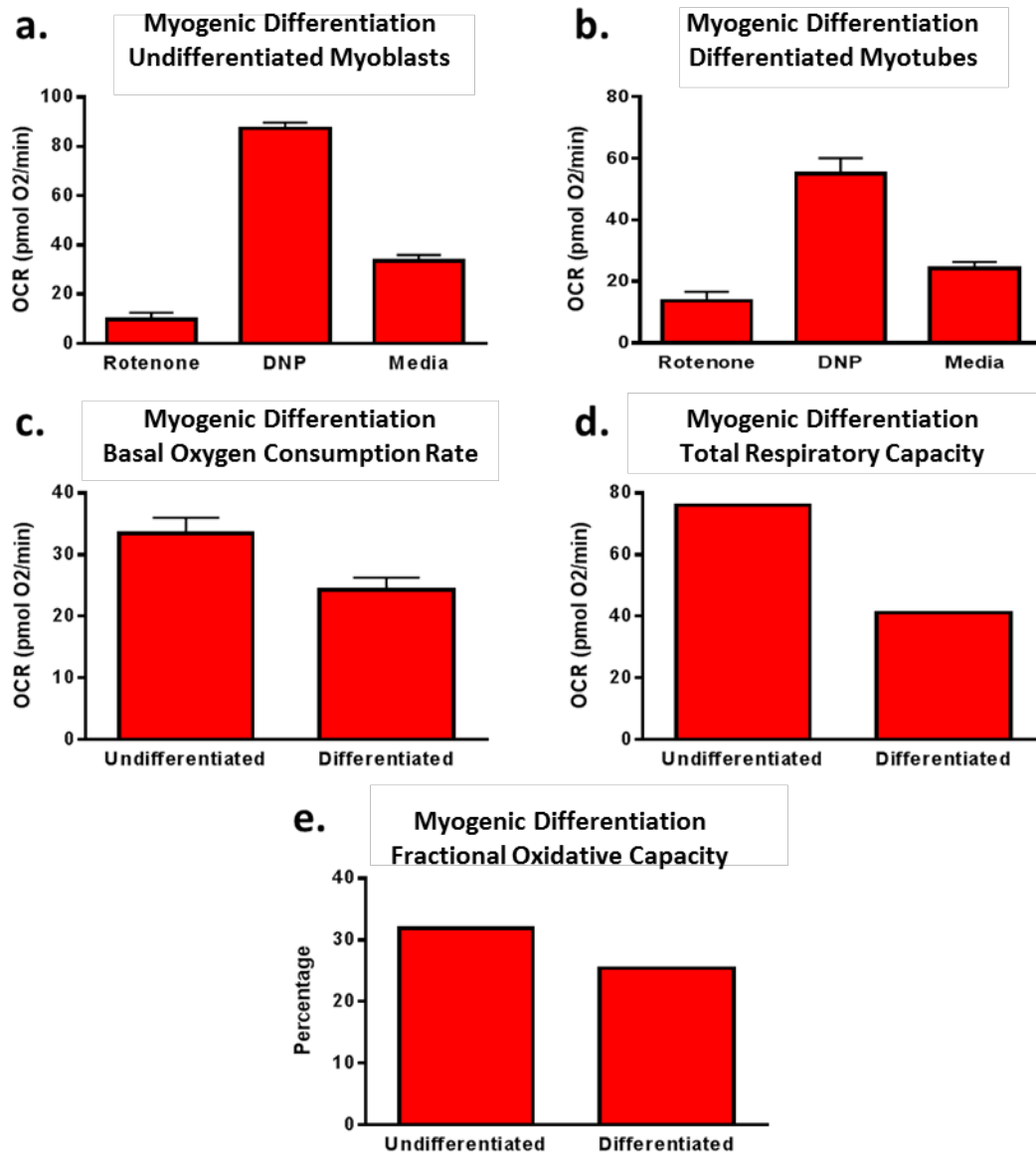
performed on differentiated myotubes. This is a good control model considering we differentiate human myogenic precursors into mature myotubes similar to differentiation of human adipocyte precursors into mature adipocytes. Similar to adipogenic differentiation we wanted to be sure our differentiation of primary human myoblasts was characterized. Differentiation demonstrated a high degree of staining for polymerized actin in multinucleated tubes (Fig. 25a) and a marked increase in expression of myogenic markers including desmin (Fig. 25b), myoD (Fig. 25c), and myosin heavy chain (Fig. 25d). When measuring these markers over time using fluorescence staining and laser scanning cytometry we found that the differentiation at least with respect to these markers plateaued around day eight post induction of differentiation and was maintained through at least day thirteen (Fig. 25e).

Oxygen consumption studies were performed on undifferentiated myoblasts and day 10 differentiated myotubes. Differentiation resulted in decreased basal oxidative rate (Fig. 26a, b), decreased total oxidative capacity (Fig. 26c), and increased fractional oxidative state (Fig. 26d). This data was different than the observations in adipocytes previously described indicating that the changes observed in our adipogenic model are not a result commonly associated with *in vitro* differentiation.





**Figure 25. Myoblast to myotube differentiation as a control differentiation system.** In vitro differentiated human myotubes stained for polymerized actin using phalloidin-AlexaFluor 488, green, and multinucleation using Hoechst 33342, blue (a), and costained with antibodies against myogenic markers conjugated to red fluor for desmin (b), myoD (c), and myosin heavy chain (d). Image analysis of fluorescence intensity normalized against nuclei for each of these markers over timecourse (e).



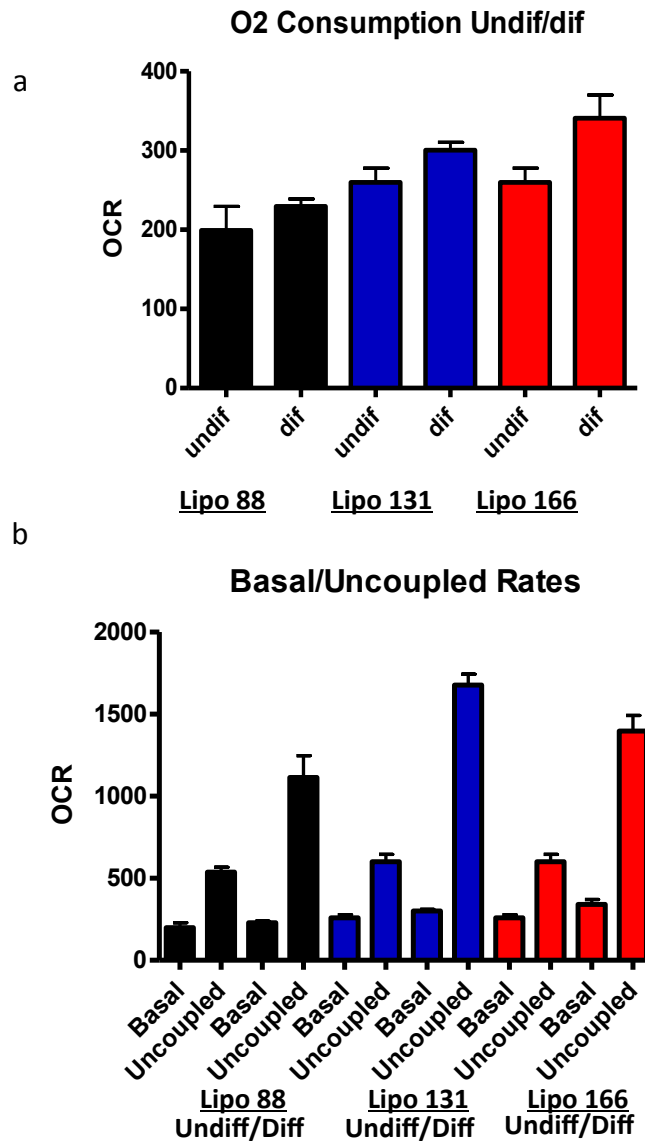
**Figure 26. Changes in basal, capacity, and fractional oxidative values in differentiating myotubes.** Oxidative data in a single donor of differentiating myotubes was obtained as described in Fig 22, 23. Comparison of undifferentiated and differentiated myotubes: Rotenone and DNP effects compared to basal oxygen consumption rates (a,b), comparison of basal rates (c), oxidative capacity (d), and fractional oxidative capacity (e).

## **B.6 Oxidative measurements associated with differentiation.**

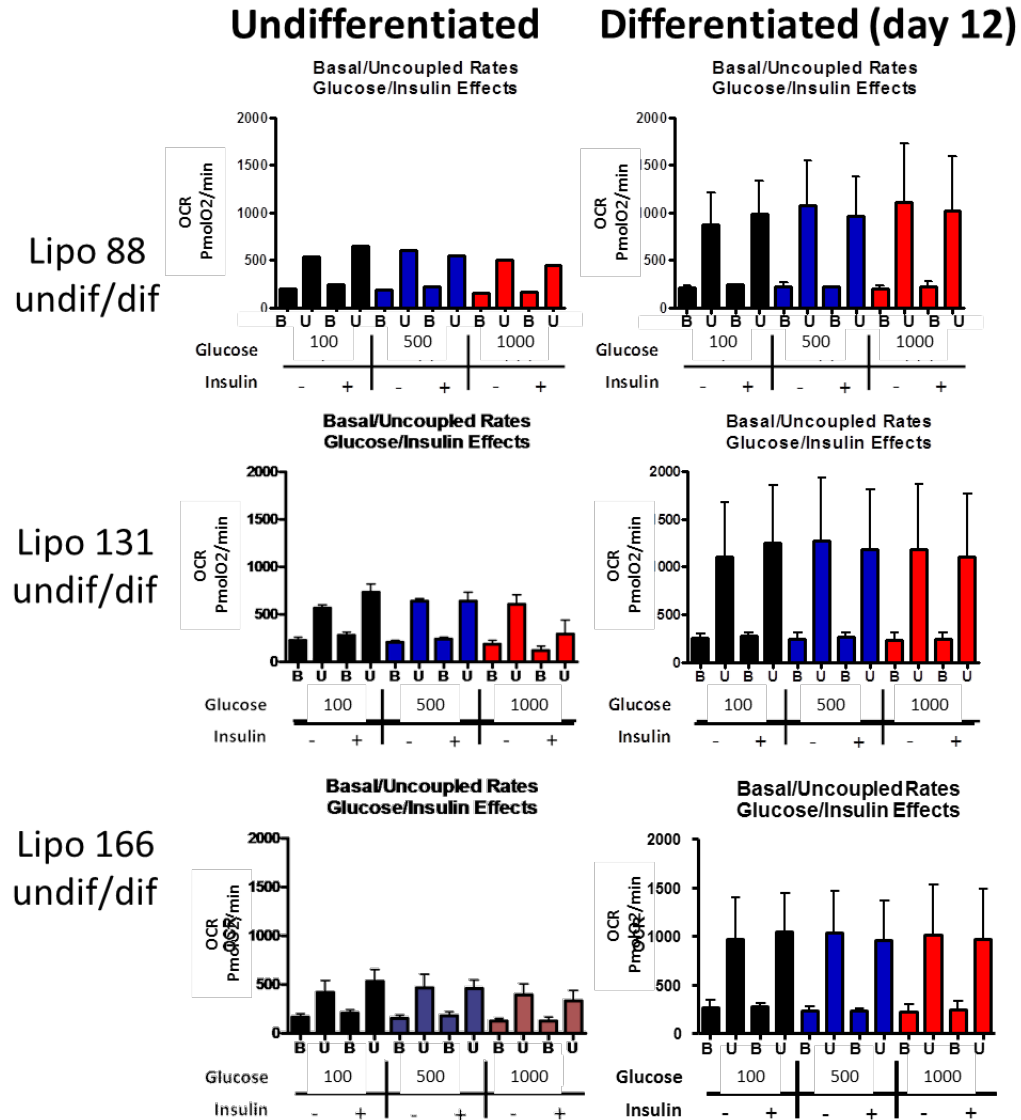
To determine the amount of oxidative metabolism occurring in the adipocytes, we measured oxygen consumption rates in undifferentiated ASCs and differentiated adipocytes using the same donors under the same conditions as the lactate measurements were made. Data indicated a small but consistent increase in basal oxygen consumption across each of the donors tested (Fig. 27a). To determine whether the basal rates observed were near saturating the oxidative capacity of the cells, basal rates were compared against uncoupled rates (DNP treated) as previously described. Each of the donors demonstrated excess oxidative capacity above basal oxidation (Fig. 27b) and again showed a larger excess oxidative capacity in the differentiated state than the undifferentiated state (Fig. 27b). This again confirmed that in each of the donors tested, the increase in lactate production did not occur because the ability to perform oxidative metabolism was saturated in the differentiated adipocytes.

Similar to lactate measurements, insulin had little to no effect on oxygen consumption rates in the undifferentiated (Fig. 28a, c, e) or the differentiated adipocytes (Fig. 28b, d, e). However, contrary to lactate data, increasing glucose concentration did not demonstrate an increase in oxidative rate in both the undifferentiated and differentiated adipocytes (Fig. 28a-e) whereas it increased lactate production only in the differentiated adipocytes. In all conditions tested, DNP stimulation identified significant excess oxidative capacity, indicating the cells are not being “forced” to perform glycolytic metabolism because oxidative processes are

saturated. Also, the increase in lactate production in differentiated adipocytes in response to increased glucose without an increase in oxidative rate suggests that excess glucose above some threshold is more likely to be metabolized with glycolytic process than oxidative process.



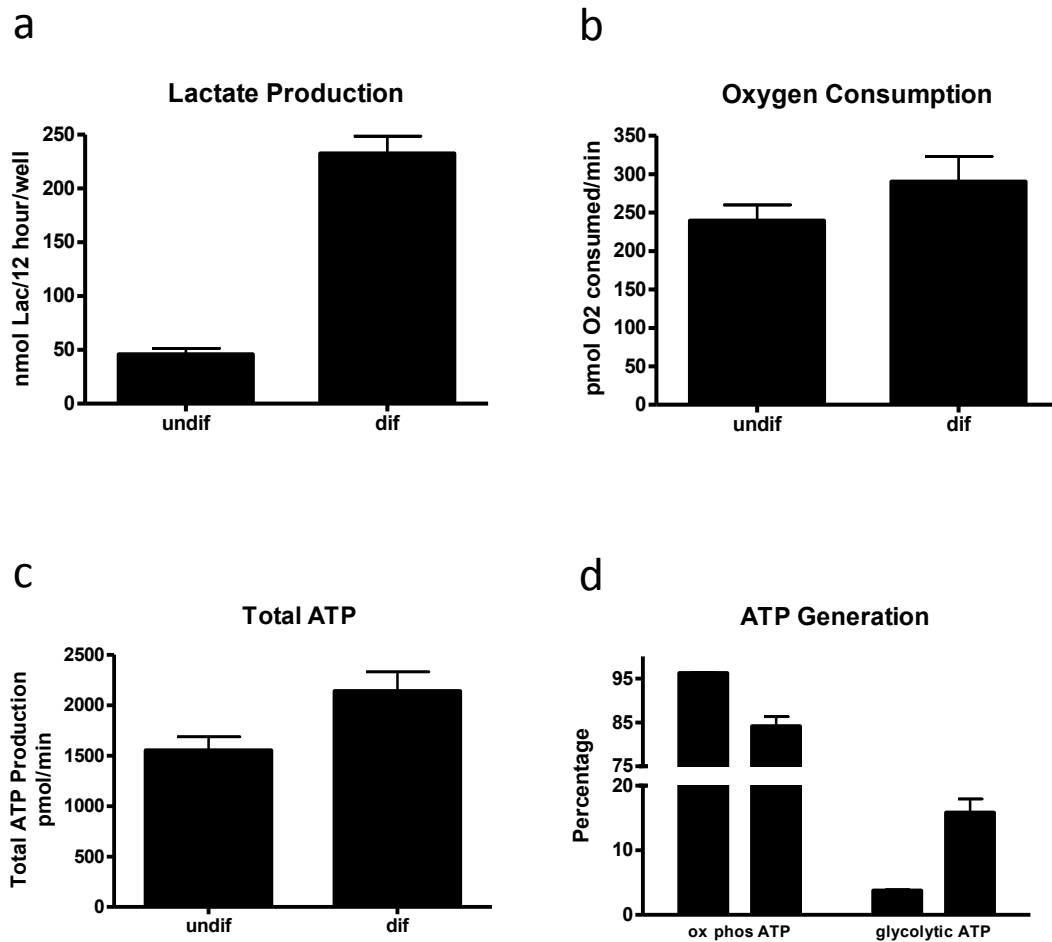
**Figure 27. Small increase in basal oxidation rates with differentiation but not near saturating capacity.** Oxygen consumption rates were measured using Seahorse as previously described. Basal oxygen consumption rates are shown for ASCs both undifferentiated and differentiated into adipocytes from three donors (a). DNP was used to compare basal and saturated (uncoupled) oxygen consumption rates in undifferentiated and differentiated cells from three donors (b).



**Figure 28. Insulin and glucose effects on oxygen consumption rates in differentiated and undifferentiated ASCs.** Basal (B) and uncoupled (U) oxygen consumption rates were measured in conditioned media as previously described on undifferentiated (a, c, e) and differentiated ASCs(b, d, f) from three donors in various hyperglycemic conditions(100, 500, 1000 mg/dl) and with and without addition of 100 nM insulin.

## B.7 Oxidative/glycolytic ratios

To better understand changes in oxidative relative to glycolytic ratios, data from all donors was combined demonstrating a 5.2 fold increase in lactate production (Fig. 29a). At the same time, differentiated adipocytes demonstrated a 1.2 fold increase in oxygen consumption rate (Fig. 29b). To determine how these changes compared to one another, ATP production associated with each of these processes was determined assuming 6.3 ATP generated per O<sub>2</sub> consumed (oxidative equation:  $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 38 \text{ ATP}$ ) and 1 ATP generated per lactate produced (glycolytic equation:  $C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + NAD + 2 \text{ ATP}$ ). There was a small and non-significant 1.37 fold increase in total ATP production from these processes combined comparing differentiated versus undifferentiated adipocytes (Fig. 29c). However, determining what percentage of total ATP was from glycolytic and oxidative processes demonstrated a significant decrease in percentage of ATP from oxidative process (1.14 fold, SEM 0.03) and a significant increase in percentage of ATP from glycolytic process (3.8 fold, SEM 0.6) (Fig. 29d). This indicates the cells exhibit a Warburg-like effect whereby the cells are increasing the fraction of substrate metabolized in glycolytic process compared to oxidative process even at “normal” atmospheric oxygen tensions.



**Figure 29. Lactate production compared with oxygen consumption rates and effects on ATP production in undifferentiated and differentiated adipocytes.** Lactate data from all donors tested is combined (a) as well as oxygen consumption rates from all donors tested combined (b). Total ATP production from these two processes is determined as described in methods and shown for undifferentiated and differentiated ASCs for all donors tested (c). Percentage of the total ATP from oxidative process and glycolytic process is shown (d).



## **B.8 Aerobic glycolysis summary:**

Differentiated adipocytes demonstrate a Warburg-like phenotype (aerobic glycolysis) characterized by elevated lactate production and increased oxidative capacity but minimal increase in oxygen consumption rates compared to undifferentiated control populations.

## **C. PDK Regulation of Aerobic Glycolysis in Differentiated Adipocytes**

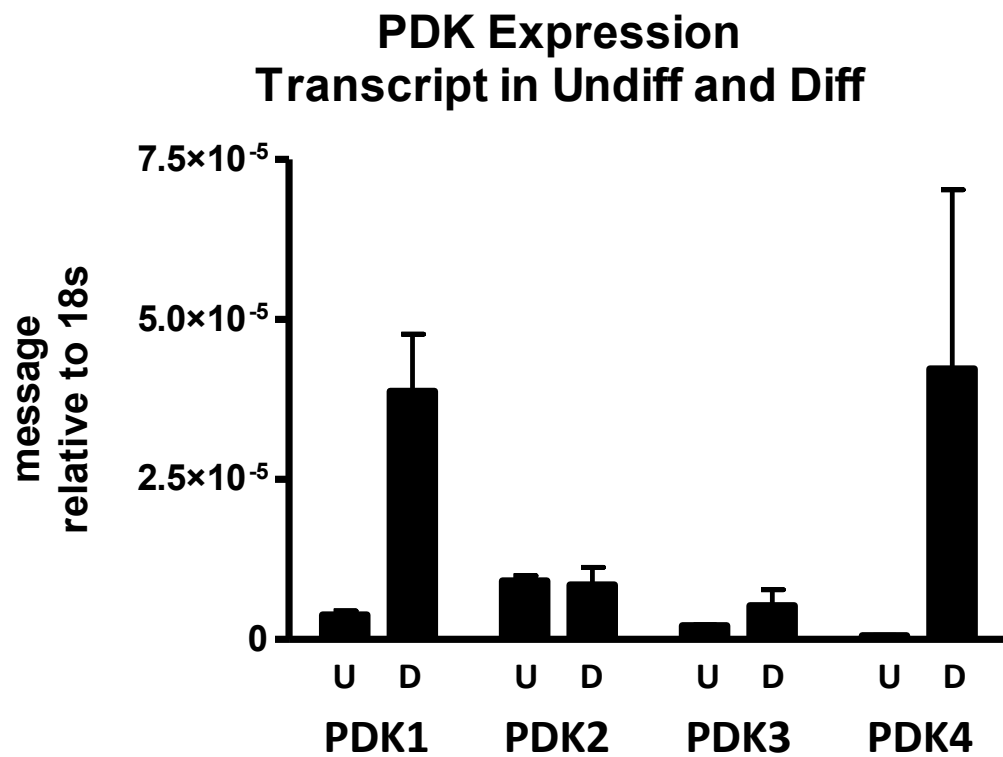
With an increase in glycolytic metabolism relative to oxidative in differentiated versus undifferentiated adipocytes the opportunity exists to test candidate regulators of the change in metabolic phenotype. The same cells display different phenotype depending on differentiation state. A change in expression of candidate proteins in differentiated and undifferentiated states potentially identifies regulators.

Pyruvate is decarboxylated to acetyl-CoA for oxidative purposes or reduced to lactate for glycolytic purposes. The regulation of pyruvate's fate is therefore a large regulator of balance between glycolytic and oxidative metabolism. PDKs phosphorylate PDH thereby inhibiting acetyl-CoA formation and increasing lactic acid fermentation and increasing the cells' reliance on glycolytic energy production. PDKs are therefore a potential regulator for the increase in glycolytic activity in differentiated adipocytes.

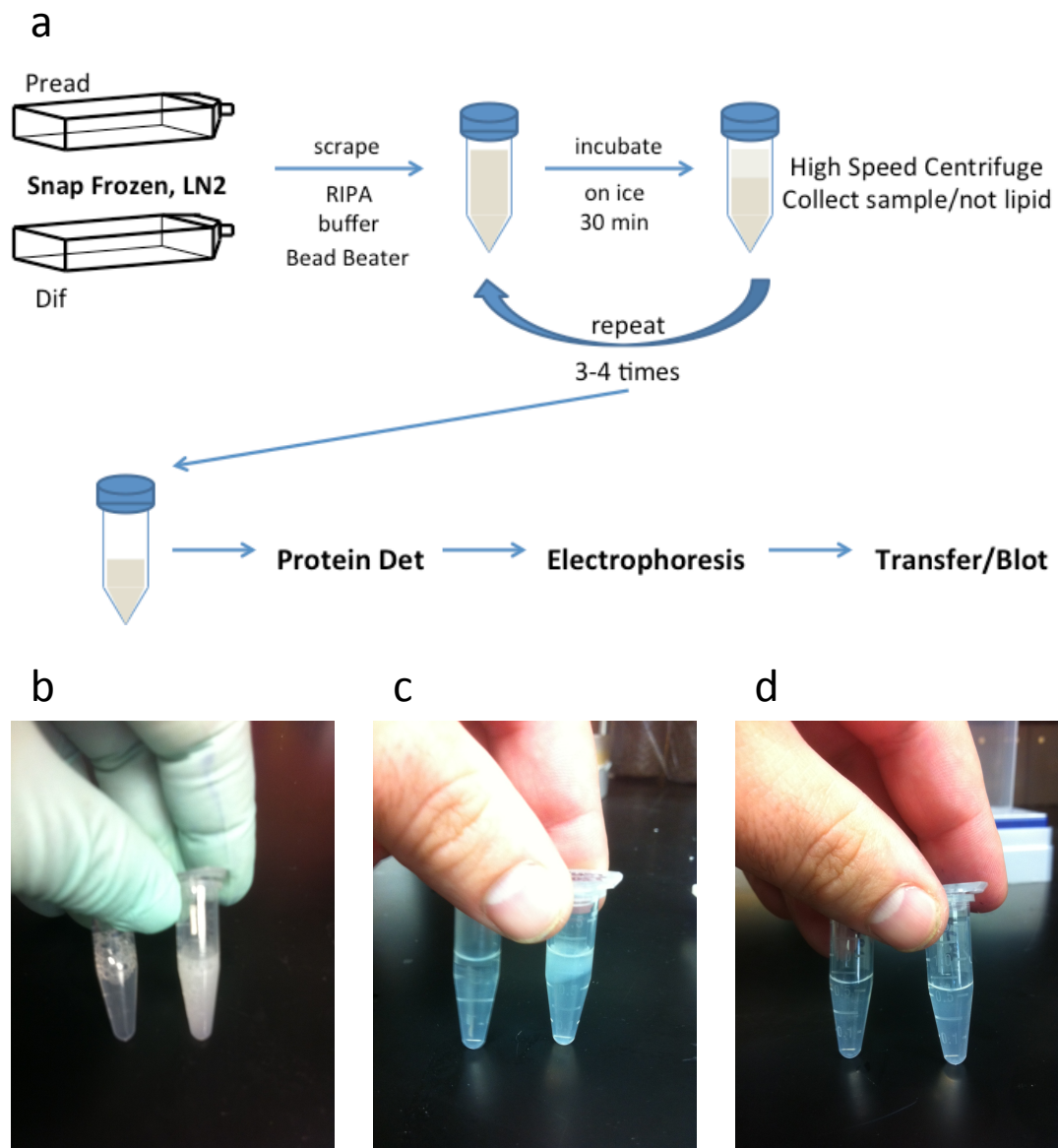
### C.1 PDK expression

RNA prepped from undifferentiated ASCs and from differentiated adipocytes was probed for transcript levels of PDK isoforms 1-4. The same three donors used in oxidative and glycolytic studies were all examined for expression changes in differentiated adipocyte state versus undifferentiated ASCs. Indeed, transcript profiling of the four PDK isoforms demonstrated an increase in PDK1 and PDK4 mRNA (Fig. 30).

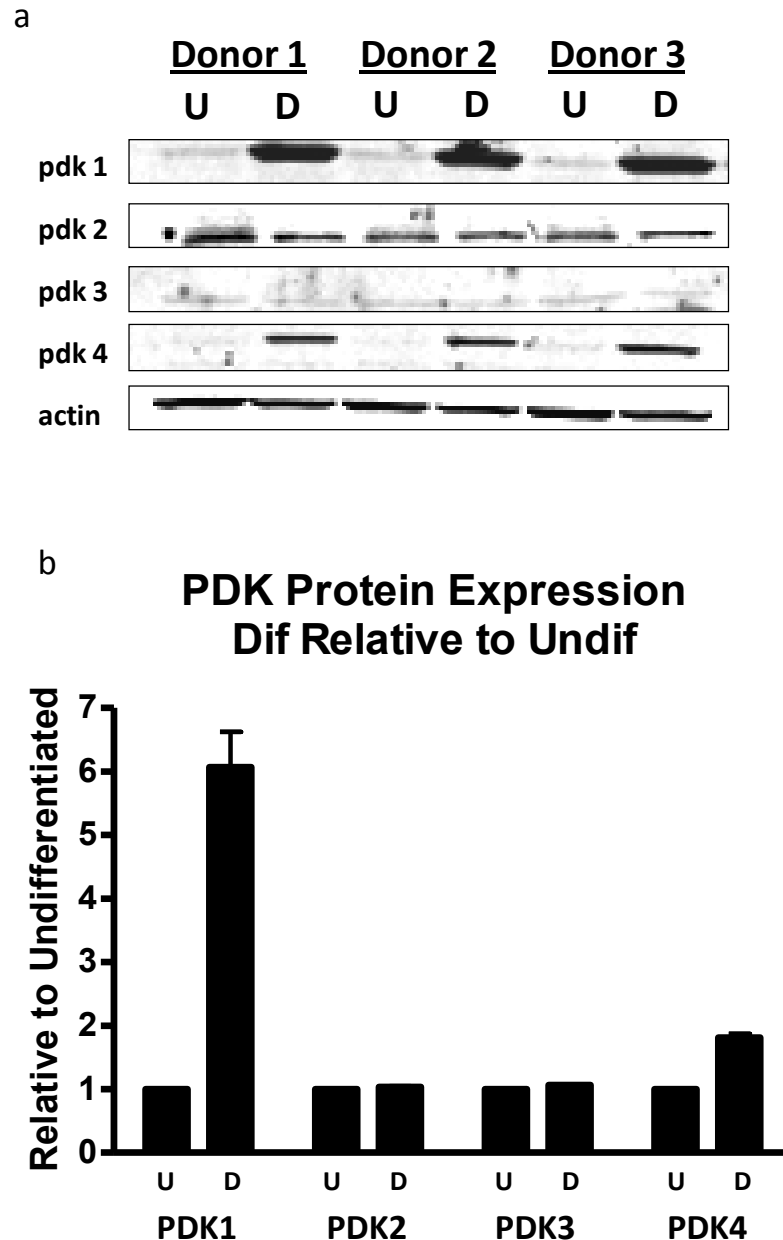
To further explore expression, protein lysates were generated on differentiated adipocytes and undifferentiated ASCs. Special care was taken to remove lipid from the samples by rounds of centrifugation and incubation on ice (Fig. 31a). The resulting samples appear to have removed their lipid content which makes protein determination and even loading difficult (Fig. 31b-d). Western blotting with antibodies for PDK isoforms 1-4 demonstrated a visible increase in PDK1 and PDK4 protein expression in differentiated adipocytes compared to their undifferentiated ASCs (Fig. 32a). Indeed, Band intensity profiling indicated a six fold increase in PDK1 and around a 1.8 fold increase in PDK4. PDK2 and PDK3 demonstrated no significant increase in western blot analysis (Fig. 32b). The increase in expression of enzymes that increase glycolytic metabolism correlated with the increase in glycolytic metabolism in the differentiated adipocytes indicating that PDKs 1 and 4 most likely play a role in regulating the Warburg-like effect observed.



**Figure 30. Increased PDK transcript associated with adipocyte differentiation.** RNA was generated from undifferentiated and differentiated adipocytes from three donors and assessed for PDKs 1-4 by Taqman. Data is average of all three donors for specific PDK isoform relative to 18s.



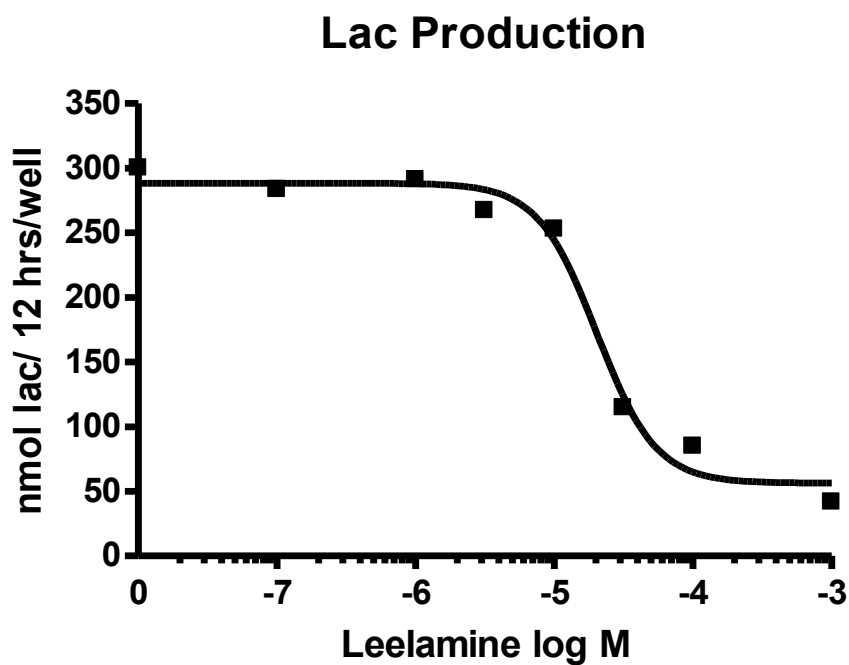
**Figure 31. Lipid removal and protein preparation from adipocyte lysates.** Schematic of protein sample preparation to remove excess lipid from samples (a). Images were taken of lysates showing undifferentiated lysate on the left and differentiated adipocyte lysate on the right showing initial lysis in RIPA buffer (b), after initial centrifugation (c), and after cleanup process/lipid removal (d).



**Figure 32. Increased PDK protein with adipogenic differentiation.** Lysates of undifferentiated and differentiated adipocytes from three donors were prepped as previously described and were analyzed by western blot for PDKs 1-4, blot images (a) and densitometry relative to undifferentiated bands is shown (b).

## C.2 Pharmacologic inhibition

To test whether the PDKs play a regulatory role in the Warburg like effect observed in the differentiated adipocytes, we employed the use of the PDK inhibitor leelamine. Leelamine treatment should reduce lactate production if the PDKs play a regulatory role in the increased glycolytic phenotype observed in differentiated adipocytes. Indeed, treatment with leelamine dose responsively decreased lactate production when tested on differentiated adipocytes from each of the donors previously used for both metabolism and PDK expression studies (Fig. 33). Inhibition of PDK resulting in a reduction of the glycolytic phenotype in differentiated adipocytes indicates that the PDKs for which expression is increased in differentiated adipocytes do play a role in regulating the observed Warburg like effect.



**Figure 33. PDK inhibition decreases lactate production from differentiated adipocytes.**

Differentiated adipocytes from three separate donors were treated with multiple concentrations of PDK inhibitor leelamine for 12 hours, subsequent lactate analysis on samples was performed as previously described to determine lactate production rates.

#### **D. Aerobic Glycolysis Protection Against ROS in Differentiated Adipocytes**

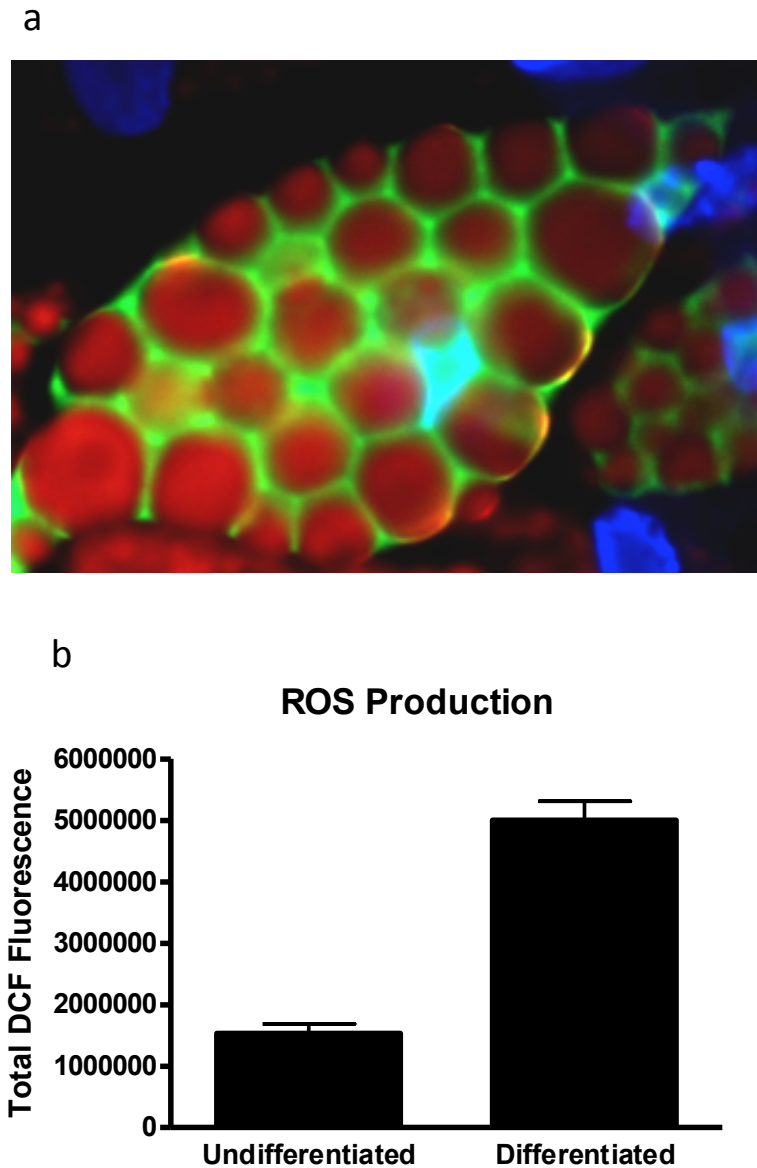
Increasing glycolytic metabolism in conditions that are permissive for oxidative metabolism is inefficient. As previously described in cancer cells, aerobic glycolysis or the Warburg effect confers protection against detrimental metabolic ROS production. To determine whether similar protection against ROS is conferred in differentiated

adipocytes by increasing metabolism via aerobic glycolysis, ROS measurements were performed.

#### **D.1 Measuring ROS in differentiated adipocytes**

As one of two key glucose depots in humans (along with skeletal muscle) increased glucose consumption in adipocytes can lead to increased metabolic ROS production. To determine if ROS production is regulated by the increase in glycolytic metabolism, differentiated adipocytes were loaded with ROS sensitive dye (Fig. 34a) and subsequently analyzed for fluorescent intensity measurements. Indeed, differentiated adipocytes demonstrated an increased amount of ROS accumulation compared to undifferentiated ASCs as seen by image and even by fluorescence intensity measurements (Fig. 34b). This increase in ROS leads to an increased need for regulation to keep ROS levels within safe ranges.

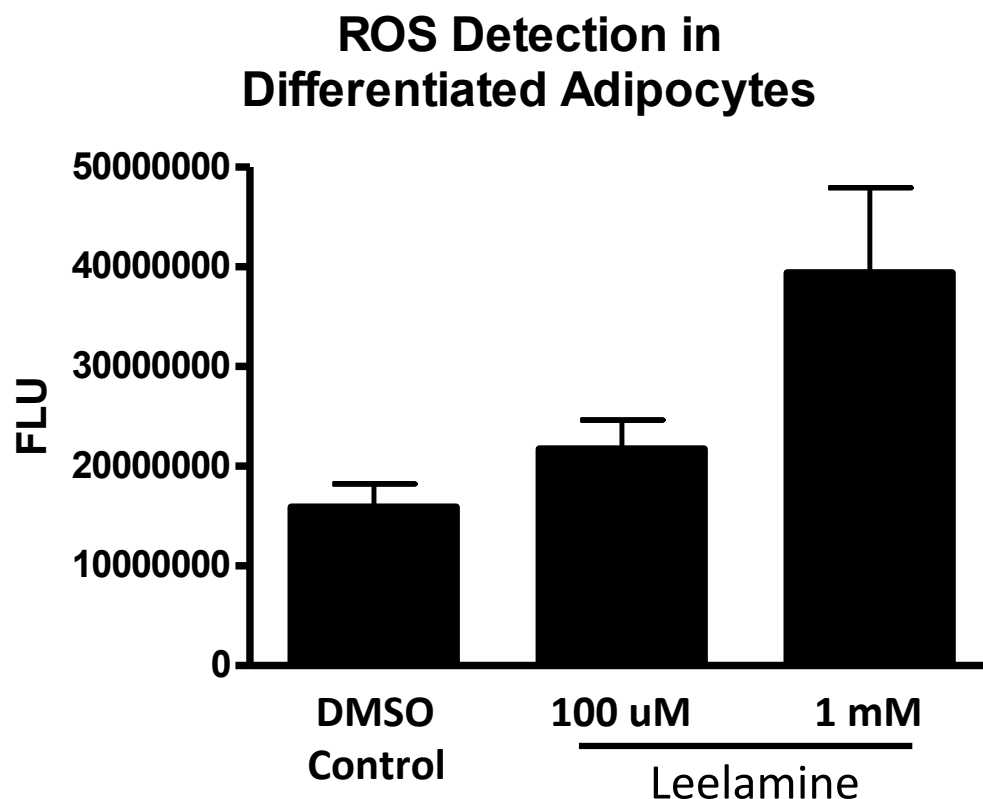




**Figure 34. ROS accumulation in differentiated adipocytes.** ASCs were differentiated as previously described and loaded with ROS sensitive dye as described in Methods. Sample image of differentiated adipocyte loaded with dye (green) and counterstained with Nile Red (red) and Hoechst 33342 (blue) (a). ROS dye accumulation analyzed by fluorescence intensity in undifferentiated and differentiated adipocytes (b).

## **D.2 PDK inhibition and ROS accumulation**

It is possible that the PDK regulated increase in glycolytic phenotype is a method the differentiated adipocytes employ to reduce metabolic ROS accumulation. To determine if this is the case, differentiated adipocytes were loaded with ROS sensitive dye and treated with PDK inhibitor (which has already demonstrated the ability to inhibit the glycolytic phenotype) and subsequent intensity measurements were performed. Treatment with varying concentrations of leelamine dose responsively increased ROS accumulation in the cells both dose dependently and over time continued accumulation for each of the donors tested (Fig. 35). The data indicated that PDK inhibition which we have already demonstrated decreases the glycolytic phenotype also results in increased ROS accumulation supporting the hypothesis that the increase in glycolysis resulting from limitation of oxidative metabolism is to keep ROS accumulation in check.

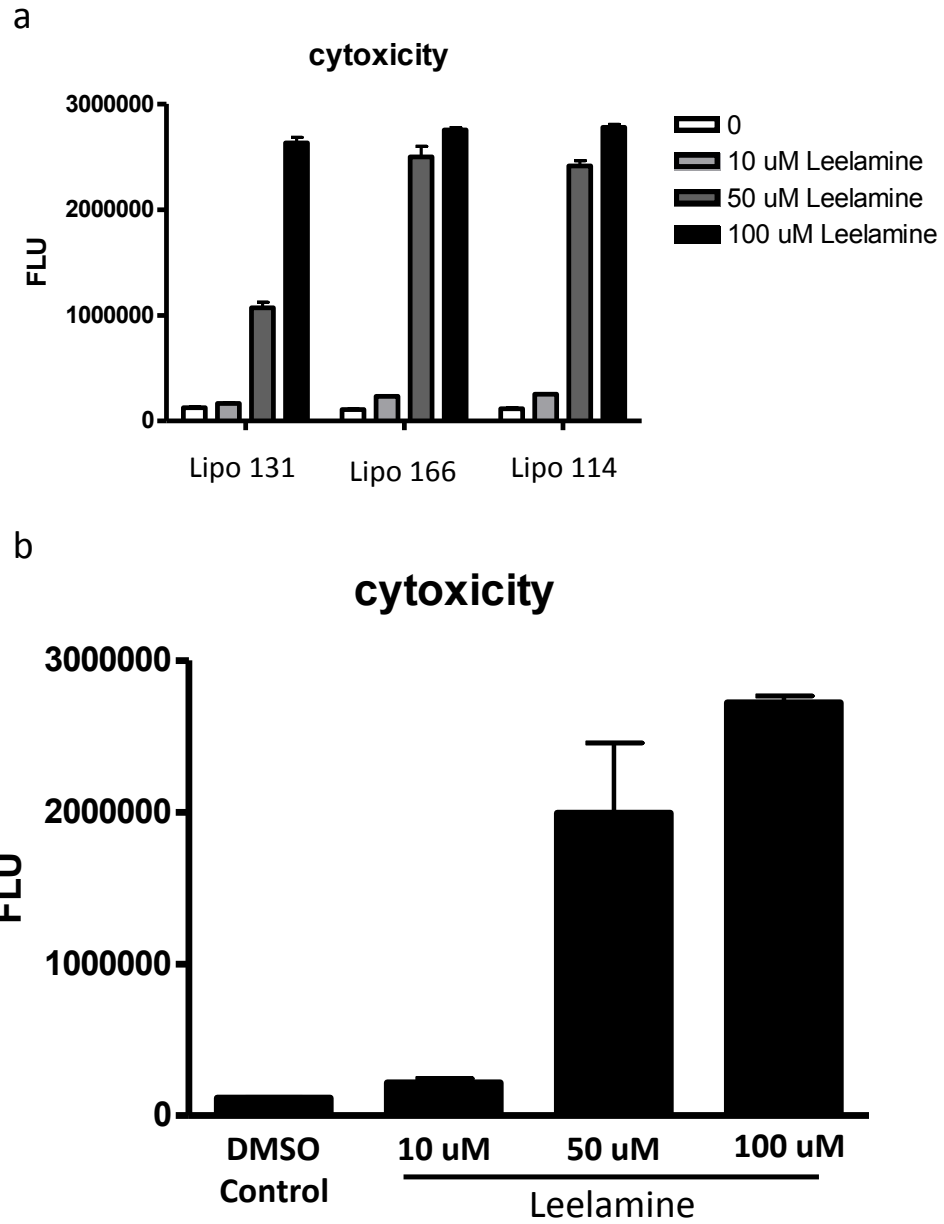


**Figure 35. PDK inhibition results in increased ROS accumulation in differentiated adipocytes.** Differentiated adipocytes from three separate donors were loaded with ROS sensitive dye as previously described and then treated with DMSO (control), 10 uM, or 100 uM Leelamine and subsequently analyzed for ROS dye after 4 hours of treatment. Data from all three donors is combined.

### D.3 Cytotoxicity and PDK inhibition

In cancer cells, the reduction of ROS accumulation achieved through aerobic glycolysis confers protection from cytolethality. To determine if the PDK regulated

glycolytic phenotype that reduces ROS accumulation serves a cytoprotective role in differentiated adipocytes, cytolethality experiments were performed. Indeed, PDK inhibition resulted in an increase in LDH release into media (Fig. 36a,b). LDH release indicates a loss of membrane integrity and is a surrogate of cytolethality. The data indicate that loss of the PDK regulated phenotype results in a loss of some cytoprotection in the differentiated adipocytes.



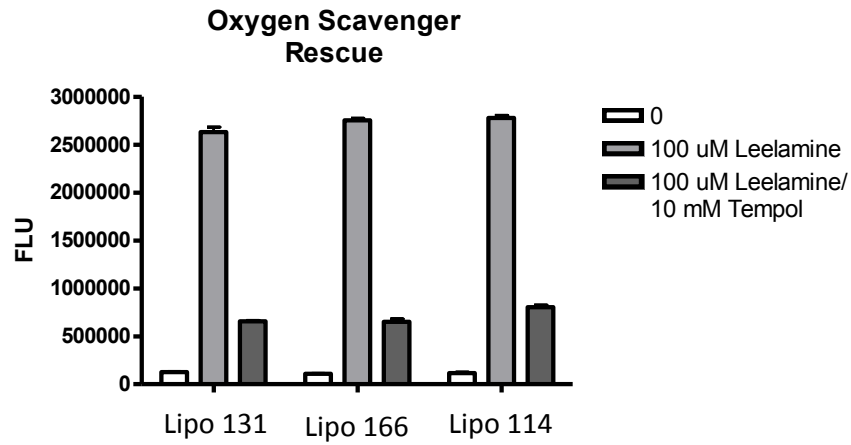
**Figure 36. PDK inhibition leads to cytotoxicity in differentiated adipocytes.**

Differentiated adipocytes from three separate donors were treated with Leelamine at control, 10, 50, and 100 uM for 36 hours. After treatment, media was collected and analyzed for LDH release by fluorescence LDH assay. Data is shown from individual donors (a) and from all donors combined (b).

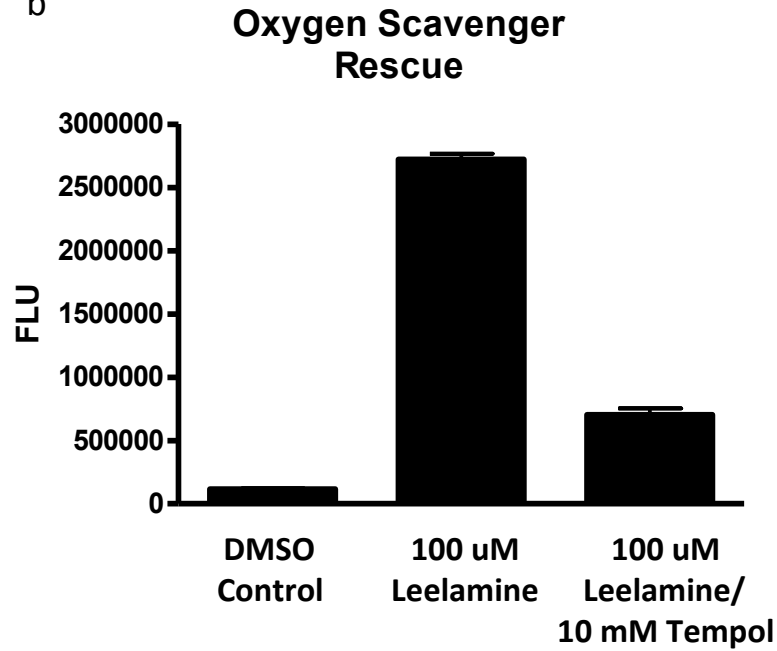
#### **D.4 PDK inhibited cytolethality is ROS mediated**

PDK inhibition resulted in an increase in ROS accumulation of the cells. It also lead led to increased cytolethality. To determine if these two were connected, cytolethality experiments were performed with ROS scavengers. If the cytolethality was due to increased accumulation of ROS in the differentiated adipocytes, treatment with antioxidant should rescue the effect. When the differentiated adipocytes were treated with both PDK inhibitor leelamine and antioxidant Tempol concurrently, LDH release from the cells was decreased (Fig. 37a,b). With rescue of cytolethality with antioxidant treatment it can be inferred that some or all of the cytolethality from leelamine treatment indeed was from ROS accumulation. This demonstrates that the PDK regulated glycolytic phenotype results in cytoprotection against ROS accumulation.

a



b



**Figure 37. ROS scavenger treatment reduces PDK stimulated cytolethality in differentiated adipocytes.** Differentiated adipocytes from three donors were treated with Leelamine at control, 100 uM Leelamine, and 10 uM Leelamine + 10 mM Tempol for 36 hours. After treatment, media was collected and analyzed for LDH release by fluorescence LDH assay. Data is shown from individual donors (a) and from all donors combined (b).

## **Chapter III:**

### **Discussion**

#### **Modeling adipogenesis**

Many adipocyte studies are done using the well established 3T3-L1 model of adipogenesis. However, this model is not a human model, rather the 3T3-L1 cells come from mouse. Beyond this, 3T3-L1 cells do not come from adipose depots or function in vivo as mouse preadipocytes for which the physiologic role would be to differentiate into adipocytes. Instead, 3T3-L1 are a sub-clone of the spontaneously immortalized 3T3 line established after 20-30 passages of mouse embryonic fibroblasts. With the goal of this thesis research focusing on human adipocyte metabolism in a context where some comparisons could be made to their precursor as a control, 3T3-L1 are a poor choice. It is much more relevant to use human cells that are the reservoir for formation of new adipocytes. For this reason, we used the adipocyte precursors (adipose stem cells or ASCs) isolated from human adipose tissue stromal vascular fraction.

Literature has demonstrated the isolation of these human adipocyte precursors (ASCs) and subsequent differentiation into mature adipocytes. To perform our planned experiments enabling the study of the glycolytic and oxidative phenotypes of differentiated adipocytes as compared to their precursors, ASCs from multiple donors



would need to be expanded and differentiated. Expansion of enough cells ex vivo for planned experiments can lead to a loss of in vivo phenotype. Considering that planned experiments depend on successful differentiation of ASCs from multiple donors it is important that the extent and lineage of differentiation is explored and understood using quantitative methods.

### **Populations used for differentiation studies are homogeneous**

When setting up a differentiation model it is important that the starting source of cells for differentiation are relatively homogeneous and devoid of contaminate cell populations. This builds confidence in statements made concerning the phenotype of the differentiated cells compared to the undifferentiated cells. The major cell types of the SVF from adipose tissue that could be mixed in with ASCs are endothelial cells and leukocytes as adipose tissue is highly vascularized and has demonstrated accumulation of macrophage and other leukocytes. The data in section 2.1.2 clearly demonstrate our starting material for adipogenic differentiation to be homogeneous and devoid of such contaminant populations. To show this, after isolation and expansion, we performed flow cytometry experiments showing no leukocytes (CD45+ cells) or endothelial cells (CD31+ cells) mixed in with the ASCs. Similarly, we demonstrated the cells were also homogeneously positive for an ASC marker (CD140b). The populations were homogeneously positive for this marker and negative for the potential contaminants from isolated and cultured stromal vascular fraction of adipose tissue. This was expected. Endothelial cells should not attach very well (if at all) to the uncoated tissue

culture treated plates we use for culture of the SVF. The leukocytes (primarily macrophage) that could attach to the plastic do not generally maintain the ability to proliferate. So the several rounds of expansion required to have enough cells for our studies would be expected to result in dilution and essentially total loss of the leukocytic population through trypsinization and splitting the cells several times with multiple doublings between these steps. While a homogeneous population was expected for these reasons, it was critical for us to perform these studies with our isolated/expanded cells to make confident whole population measurements and assertions about differentiated and undifferentiated cells.

#### **Populations used for differentiation maintain *in vivo* bi-potentiality**

Differentiation of adipocyte precursors into mature adipocytes is best achieved using high density seeding. With a large number of studies planned to assess adipogenic differentiation lineage commitment and capacity, metabolic phenotype of differentiated adipocytes and their precursors, transcript and protein expression studies, pharmacologic pathway interrogation studies, ROS generation studies, and viability studies all under multiple conditions, expansion of isolated SVF had to be performed. With expansion, primary cells often lose their *in vivo* phenotype. In particular, precursor and stem cells often lose their plasticity.

It is important to demonstrate this plasticity is maintained in differentiation models. As with all cell based modeling, the ultimate goal is to make assertions about physiology, that is, the model developed should mimic *in vivo* physiology as best as

possible. For differentiation studies, it is therefore critical that the cells being used to mimic physiologic differentiation demonstrate physiologic plasticity as best as possible post isolation and expansion. ASCs have demonstrated a bi-potential role *in vivo* (discussed in the Introduction) that includes the formation of new adipocytes and support of local vasculature. The first is a clear differentiation event from ASC into adipocyte, the second potentially involves some differentiation into a more mature vasculosupportive cell, though *in vivo*, the ASC may be performing this role even in an undifferentiated state. The data in section 2.1.3 demonstrate maintenance of this bipotentiality *in vitro* after expansion and are further discussed below.

When we placed the expanded ASCs under adipogenic conditions after expansion, the isolated and expanded ASCs clearly demonstrated maintenance of plasticity for differentiation down this lineage as seen by lipid accumulation and morphologic changes. The undifferentiated ASCs also demonstrated the ability to support vascular networks as demonstrated by cord formation of HUVECs when co-cultured with the ASCs. In co-culture, the ASCs that were juxtaposed to the forming cords and in immediate contact with the endothelium demonstrated enhanced expression of SMA further indicating the vasculosupportive role of the ASCs was maintained even after expansion. An increase in SMA was even observed in VEGF-treated populations. This further speaks to the concept that ASCs do have some maturation into an even more vasculosupportive cell type when directly contacting newly forming vessels or at the very least contacting naked endothelium. The ASCs lack KDR (VEGF receptor) and therefore must have some cross-talk with the endothelium to

increase SMA expression in response to VEGF. Though not the focus of this particular thesis, our lab has made and continues to make significant progress investigating this angiogenic event, the role of ASCs in this process *in vitro* and *in vivo*, and how this can potentially benefit cell therapy. It does however clearly demonstrate the maintenance of vasculosupportive plasticity post expansion. These studies further build confidence in the maintenance of normal physiologic phenotype with respect to differentiation capacity of the populations we use to model adipogenesis and therefore makes us more confident in our adipogenic model with respect to maintenance of normal physiology *ex vivo*.

#### **Quantitative method development for adipogenesis**

Differentiation and lineage tracing is often assessed at checkpoints using markers that attempt to demonstrate cells are changing from some stem cell or precursor into a different or more mature cell type. The cues (growth factors/metabolites/ligands/etc) provided to direct cells down a specific differentiation lineage attempt to focus the plasticity of the starting cells to progress a specific way, however, it is possible that the cells will not follow this path. Therefore, when setting up a differentiation model it is critical to demonstrate that differentiation is indeed occurring and it is progressing down the appropriate/desired lineage.

For long term differentiations or for complex transdifferentiations, this very often involves many steps and multiple checkpoints can be assessed. This is commonly done with maturation of embryonic or induced pluripotent stem (IPS) cells as multiple

steps of maturation often must be achieved systematically mimicking embryologic and/or neonatal differentiation. For progenitor or precursor maturation this is often much more simple as the final step of differentiation is occurring. This is the case for adipogenic differentiation of human ASCs. The cells have already progressed from embryonic differentiation through mesenchymal lineage commitment all the way to formation of the adipocyte precursor *in vivo*. We believe this is a benefit of using primary-sourced precursors. Mimicking differentiation *ex vivo* will never perfectly mimic differentiation *in vivo* as each step of differentiation introduces potential derivation from normal cellular development. For this reason working with precursors who have undergone most of their development/differentiation *in vivo* and only requiring final steps of differentiation *ex vivo* is beneficial. Differentiation of ASCs into adipocytes is a terminal step of differentiation that we perform *ex vivo*. While there are not a large number of milestone checkpoints to achieve in this terminal differentiation, the cells do demonstrate the ability to differentiate down alternative lineages other than adipocytes (as mentioned in the Introduction) and therefore it is important that we have a method of tracing differentiation into adipocytes.

For adipogenic differentiation, the most common method of demonstrating successful differentiation is based upon lipid accumulation in the cells. The mature adipocytes demonstrate a lipid laden morphology compared to their undifferentiated precursors. Therefore, most models of differentiation identify this lipid accumulation by staining lipids within the cell a red color using Oil Red O and subsequent microscopy. This is however poorly quantitative if quantitation is applied to this at all. As we are

using multiple donors to look at metabolic changes in mature adipocytes and we are using their undifferentiated precursors as controls, it is important that we have a quantitative method for assessing adipocyte differentiation across these donors to account for potential variability in differentiation from donor to donor. Standard Oil-Red-O staining is not quantitative enough for such an assessment. To do so we developed our own quantitative method for assessing lipid accumulation in whole populations of cells as they differentiated.

The method we developed is a quantitative imaging based approach whereby lipids are stained with a lipophilic fluor with subsequent fluorescence intensity determined using laser scanning cytometry to assess total lipid accumulation in the cells specifically within the lipid droplets. Following this, lipid is extracted, and nuclei are labeled and intensity assessed using a nuclear dye. Detailed methods development and optimization for this is described in section 2.1.4. Laser scanning cytometry enables rapid image capture and analysis of whole populations as opposed to representative individual scan areas commonly used by microscopy and standard automated imaging. This allowed quantitation of lipid accumulation and normalization to nuclei for whole population analysis as opposed to fractions of the population that could be obtained by standard fluorescence imaging. It also enables segmentation and subsequent lipid quantitation only from lipid droplets eliminating non-specific fluorescence which can't be achieved with fluorescence plate readers attempting to make whole population analysis. This method enabled fixation and analysis of samples throughout time course of differentiation to assess donor to donor variability and the effects of changing

protocol on progression through differentiation. This method can be employed in other areas of biology to quantify lipid accumulation and can be altered to look at lipid accumulation in other cell types. For example, lipid accumulation in hepatocytes can be quantified using this technique to model steatosis. Similarly, monocytes turning into foam cells are characterized by lipid accumulation in their cytosol. Development of this technique has been applied to such studies as well.

### **Assessing subcutaneous and omental adipogenic differentiation**

Employing the quantitative method in our studies of adipogenic differentiation demonstrated a clear and complete differentiation of adipocytes using lipid accumulation as a surrogate. Time-course data in section 2.1.5 demonstrate a plateau of differentiation using this approach around day 9 and extending through days 12-15 of differentiation. The cells from this point on continue to coalesce lipid droplets however, cells appear to be lost past this point. We believe the cells become buoyant, lift off the surface of the culture plate or stretch and strain the membrane too far in 2-dimensional culture and die (observations under microscope). When comparing differentiation of omental preadipocytes to subcutaneous preadipocytes, we saw reduced levels of differentiation in the omental cells. This was expected based on previous work in the field, however, limited numbers of omental versus subcutaneous preadipocyte differentiation studies have been performed in the past (none with a quantitative method such as ours) and thus we were compelled to use our quantitative approach to study this with a single donor, matched-pair of omental and subcutaneous

preadipocytes from the same donor. Using matched donor adipocyte precursors from separate adipose depots reduce any genetic or epigenetic variation that could contribute to similar studies comparing omental and subcutaneous ASC differentiation when the separate donors are used to source cells from the different depots.

As expected, PPAR gamma agonist improved differentiation in subcutaneous and omental adipogenic differentiation. However, even with PPAR gamma agonism throughout the time course of differentiation, sufficient differentiation was not achieved using omental derived ASCs to model adipogenesis and make assertions about metabolic phenotype in differentiated adipocytes vs undifferentiated precursors when whole populations measurements would be made. The differentiated population was too heterogeneous of a mix of undifferentiated and differentiated adipocytes. As a result, our continued studies primarily focused on differentiation of subcutaneous adipocytes.

### **Donor to donor variability**

In sections 2.1.6-7 we demonstrate consistency across multiple donors. As previously discussed, to better assess human adipocyte metabolic phenotype, we used primary cells from human adipose tissue (ASCs). Single donor/single source studies provide valuable information; however, there is the potential that observations made from such studies are due to some unforeseen genetic or epigenetic predisposition of the particular donor being used. Pooled lots of undifferentiated cells can be used, however, often one particular component donor of the pooled lot may predominate



over the others depending on the function being tested or phenotype/genotype being studied. To account for this, we tested multiple donors for critical measurements being made throughout this thesis.

In assessing variability in differentiation capacity, our data demonstrated a very consistent differentiation across 8 separate donors (plus superlot) when tested for lipid accumulation over time. Indeed, the majority of lipid accumulation/morphologic change was observed in nearly all donors tested between days 3-6 of differentiation. As this method was newly developed in our lab, alternatively we confirmed these results measuring intracellular glycerol accumulation as a more standard surrogate of triglyceride accumulation, the preferred method of lipid storage in adipocytes. This helped confirm the more high content novel approach we developed and further demonstrated through a separate measure that the cells were differentiating with little variability across donors.

While lipid accumulation is often used to demonstrate adipogenic differentiation we demonstrated a second non-lipid measurement to show adipogenic lineage differentiation. This was achieved by measuring adipokine production from the cells throughout differentiation. This is important as multiple cell types can lipid load (hepatocytes, macrophage, skeletal muscle, etc) but will not produce these adipokines. Our data demonstrated a minimal variation amongst multiple donors tested for production of leptin and adiponectin. Production of both adipokines increased throughout differentiation. Interestingly, adiponectin precedes leptin accumulation in

the media. Adiponectin is perhaps more sensitive to the PPAR gamma agonist that is added during the early stages of differentiation. This is consistent with literature which identifies adiponectin as a biomarker of PPAR gamma agonist treatment *in vivo*. This data (adipokine production) further validates appropriate adipogenic differentiation and demonstrates (as does lipid accumulation analysis) that donor to donor variability is very low in our model. And it makes us confident that metabolic measurements from multiple human donors would not be skewed by variability in differentiation between donors.

### **Lactate production and aerobic glycolysis in differentiated adipocytes**

As discussed in the Introduction it has been shown that adipose tissue generates lactate. But how this lactate is generated, and what is regulating its production is still unknown. Using our differentiation model, we were able to demonstrate similar lactate production from the mature/differentiated adipocytes and not in their precursors. This identified the precursors as an ideal control to study regulation of this lactate production in the differentiated adipocytes.

Initial metabolism studies on multiple depots shown in section 3.2 identified increased glucose consumption coupled with an increase in lactate production. This was our initial data suggesting there may be a Warburg-like effect occurring in differentiated adipocytes compared to their precursors. As previously introduced, the Warburg effect or aerobic glycolysis is an increase in lactate production in normal oxygen tensions. The cells in our human adipocyte model are maintained in atmospheric oxygen throughout

differentiation (21%). The increase in glucose consumption was expected as the cells increase expression of Glut1 during adipogenic differentiation, Glut4 is also increased, however it is primarily only active with insulin stimulation and resulting insertion into the membrane. The increase in glucose consumption helps the differentiating cells store fatty acids by providing substrate for glycerol production (glyceroneogenesis as described in introduction). It also is part of the new energy storage/glucose clearance role the differentiated cells have compared to their undifferentiated precursors. The elevation of lactate production indicates that some portion of the glucose was being metabolized through lactic acid fermentation. Again, at normal oxygen tensions, this was indicative of aerobic glycolysis occurring in these cells. It also points towards the production of lactate in adipose tissue may not be due to hypoxia in the tissue or intermittent hypoxia in the tissue but because of the metabolic phenotype of the adipocytes. Further studies in human would need to be performed to better correlate realtime lactate production with oxygen tension measurements in the tissue.

While the same trends (increased glucose consumption coupled with increased lactate production) were seen in both the subcutaneous and omental differentiation models, we are more confident in statements about the subcutaneous model as previously discussed due to the heterogeneity of the omental cells by the end of differentiation. Seeing the similar trend indicates however, that the cells that are differentiating in that particular model are most likely demonstrating similar metabolic changes and may indicate similar progression into aerobic glycolysis in both omental and subcutaneous adipocytes. However, further studies for this thesis research

focused on subcutaneous adipocytes where differentiation produced a more homogeneous population of differentiated adipocytes as previously discussed.

The preliminary data were confirmed when tested on multiple donors. Lactate production increased an average of 5.2 fold across donors in differentiated adipocytes compared to their undifferentiated precursors (with each donor significantly increasing lactate production in differentiated state). Again, this increase in glycolytic metabolism was observed in normoxic conditions demonstrating aerobic glycolysis for all donors tested. This metabolic phenotype was held up under all conditions tested (increased glucose concentration and stimulation with insulin). Differentiated cells even demonstrated an increase in lactate production when glucose concentration was increased. We believe this indicates that increased glucose was being glycolytically metabolized. This demonstrates that the model was behaving similar to clinical studies of human adipose tissue mentioned in introduction where adipose lactate production increased after feeding and may suggest excess glucose beyond some concentration may be preferentially metabolized glycolytically (discussed further later).

Taken together, the lactate data indicate that in all donors tested a Warburg-like effect was occurring in the differentiated adipocytes compared to their precursors characterized by increased lactate production at normoxic conditions. This also confirmed the differentiated adipocytes were metabolically similar to what has been observed in clinical studies of human adipose tissue where lactate production occurs

and suggests that this production of lactate is potentially part of normal adipocyte metabolism.

### **Oxidative metabolism and aerobic glycolysis in differentiated adipocytes**

To better understand metabolic changes and characterize aerobic glycolysis, we performed oxidative metabolism measurements (data in section 2.2.4). Oxygen consumption rate (OCR) measurements were taken as a surrogate of oxidative activity in the cells. Oxygen consumed is primarily being used in the electron transport chain and therefore its rate of consumption is directly related to oxidative metabolism. This was confirmed by inhibition of site 1 of electron transport with rotenone and a resulting decrease of OCR to near non-existent levels. Comparing differentiated to undifferentiated adipocytes demonstrated only a very small increase in oxidative metabolism in the differentiated cells.

This small increase in oxidative metabolism is interesting as we saw large increases in lactate production (glycolytic metabolism). Such data further confirms the existence of an aerobic glycolysis phenotype in the differentiated cells compared to their precursors. This data is similar to what Otto Warburg initially found in tumor models. Cells displaying the Warburg effect do not demonstrate decreased oxidative metabolism, rather they demonstrate increased lactate metabolism, in effect increasing the amount of ATP generated from glycolytic processes compared to oxidative processes even though measurements are being made in normoxic conditions. Our data certainly supported this when theoretical ATP yields were calculated from glycolytic and

oxidative measurements (Section 2.2.6). These demonstrated that with differentiation, overall ATP production increases. Both oxidative and glycolytic metabolism show an increase in ATP production, however, as a percentage of contribution to total theoretical ATP production, glycolytic metabolism increases whereas oxidative metabolism decreases. Again, these calculations clearly demonstrate existence of the Warburg effect or an aerobic glycolysis phenotype in the differentiated adipocytes.

It is also important to note that there were no increases in oxidative metabolism in the same glucose and insulin conditions previously discussed for lactate measurements. That is, increasing glucose did not result in increased OCR. This is particularly interesting as lactate production did increase with glucose concentration in the differentiated adipocytes. This is perhaps indicating that there is some threshold level of glucose flux and metabolism above which excess glucose is increasingly glycolytically metabolized though further studies with more glucose concentrations for lactate production and OCR measurements would need to be performed to confirm this.

#### **Oxidation in mitochondria is not rate limiting.**

Using mitochondrial toxins we were able to measure maximum and minimum OCRs with respect to mitochondrial metabolism. By doing so, we were able to determine total oxidative capacity (TOC) and fraction of oxidative capacity or a measure of how much of the oxidative the capacity the cells were using. These calculations are described in results. Briefly, minimum oxygen consumption was determined by treatment with rotenone which inhibits site one of electron transport, inhibiting

oxidation in the mitochondria; maximal oxygen consumption was determined by allowing proton movement across mitochondrial membrane using the proton ionophore DNP resulting in maximal oxidation in an attempt by the mitochondria to restore the proton motive force. Interestingly, though there was only a modest increase in basal oxidative metabolism in the differentiated cells compared to their precursors, their TOC increased robustly (section 2.2.4). As a result, even though there was a small increase in basal oxidative rate, as a function of what the cells were capable of the fraction of oxidative capacity decreased with differentiation. In other words, the ability to perform oxidative metabolism increases considerably with adipogenic differentiation; however, the increase in oxidative capacity far exceeds that actual amount of oxidation occurring. As we demonstrated in section 2.2.5, performing similar oxidative measurements in differentiated human myotubes compared to their undifferentiated precursor myoblasts as a model of myogenesis does not demonstrate the large increase in oxidative capacity that accompanies adipogenic differentiation. This indicates that the observed changes in oxidative capacity and percentage of that capacity being used in the adipocytes is not some event common to differentiation models *in vitro*.

Measuring oxidative capacity and especially observing DNP stimulated maximal OCR was important to demonstrate that increases in lactate production were not due to saturation of oxidative processes thereby causing a build-up of pyruvate enhancing lactate dehydrogenase action. In other words, this data indicates that the cells are capable of oxidizing more of their pyruvate, they are instead performing lactic acid fermentation, not simply because they are “forced” to do so by limited oxidative

capacity. This points towards the concept that the cells are purposefully increasing their glycolytic metabolism via a controlled mechanism in the differentiated adipocytes and not in the undifferentiated cells (where aerobic glycolysis is not observed).

### **PDK regulation of aerobic glycolysis in differentiated adipocytes**

With the increase in glycolytic metabolism observed in differentiated adipocytes and not in their undifferentiated precursor state, we were able to test for changes in expression of candidate regulators of glycolytic vs oxidative metabolism. The PDKs, as already introduced, are an interesting class of these regulators. They are targeted in tumor cells to inhibit aerobic glycolysis and have demonstrated a role in regulating glyceroneogenesis in adipocytes so there is precedence that these are expressed in adipocytes and are potentially involved in regulation of aerobic glycolysis in our model. Data in section 2.3.1 demonstrated increased expression of PDK1 (and to a lesser extent PDK4) in the differentiated cells compared to undifferentiated precursors indicating correlation of increased expression where aerobic glycolysis is occurring. Indeed, PDK activity would induce a build-up of pyruvate by diminishing its conversion to acetyl-CoA by PDH slowing down PDC activity by phosphorylation. The increase in expression of PDKs in the differentiated state point to the concept that differentiated adipocytes are programmed to run a more glycolytic metabolism in their differentiated state. More simply put, the data suggest that part of adipocyte maturation is an alteration of metabolic phenotype that enhances the ability to perform aerobic glycolysis. This agrees with our previous data indicating the cells are not forced to increase lactic acid



fermentation due to a rate-limit in oxidative metabolism. Increased expression of PDKs correlated with increased aerobic glycolysis in differentiated adipocytes further supports that the ability to demonstrate the Warburg effect is associated with normal adipocyte maturation.

Pharmacologic inhibition of PDKs in our model better demonstrated their role in regulating the increase in glycolytic metabolism. Inhibition with Leelamine has demonstrated PDK inhibition in adipocyte models to study regulation of glyceroneogenesis. Leelamine inhibition of PDK in our model demonstrated dose-dependent reduction in lactate production. In effect, inhibiting PDK resulted in loss of the glycolytic phenotype (as characterized by decreased lactate production with Leelamine). This data further supported the role of PDKs in regulation of aerobic glycolysis in differentiated adipocytes.

The correlation of increased PDK expression with the aerobic glycolysis phenotype and loss of that phenotype with pharmacologic inhibition of PDK demonstrate a regulatory role for PDK controlling the observed aerobic glycolysis in differentiated adipocytes. While adipose tissue has demonstrated lactate production in clinical studies, how this is regulated at a cellular level has been poorly understood, we believe our data experimentally demonstrates such a regulatory role for PDKs in adipocytes.

## **Aerobic glycolysis and ROS production in differentiated adipocytes**

As previously mentioned in the Introduction, ROS regulation in adipocytes is critical for maintaining normal adipocyte function and viability. As previously discussed in introduction, tumor cells are susceptible to ROS damage and demonstrate aerobic glycolysis to reduce oxidative stress. The proposed mechanism is that by switching some glucose metabolism to lactic acid fermentation, the amount of oxidative metabolism that would be occurring is reduced, and with this reduction in oxidative metabolism, mitochondrial ROS production is decreased. Our data indicates a similar purpose for aerobic glycolysis in differentiated adipocytes to reduce or regulate ROS production.

We have already demonstrated the existence of a metabolic phenotype in differentiated adipocytes characterized by an increase in aerobic glycolysis. This can be inhibited when treated with PDK inhibitor Leelamine. When we treat with Leelamine and inhibit aerobic glycolysis in the differentiated adipocytes, we also observe an increase in ROS accumulation in the cells. Similar effects are observed in tumor cells when inhibiting PDKs. The elevated ROS production is most likely mitochondrial sourced as PDK inhibition allows more pyruvate conversion to acetyl-CoA and subsequent oxidative phosphorylation. Our data point towards the potential role of a PDK regulated Warburg effect in differentiated adipocytes to help control ROS production. To be more definitive with this, oxidative metabolism studies need to be performed demonstrating increased OCR with PDK inhibition need to be performed that correlate

with the increase in ROS production and reduction of lactate production. However, from our data, it is clear that inhibition of the aerobic glycolysis increases ROS production in differentiated adipocytes.

### **Aerobic glycolysis and cell health in differentiated adipocytes**

As mentioned previously, tumor cells perform aerobic glycolysis as a cytoprotective mechanism against the detrimental effects of ROS. Indeed, PDK inhibition resulting in loss of the Warburg effect in tumor cells has demonstrated cytotoxicity. Our data demonstrates that a similar role is most likely occurring in differentiated adipocytes. When we inhibit aerobic glycolysis in the differentiated adipocytes, we see an increase in ROS accumulation over the first several hours; however, the cells appear healthy and viable morphologically. When we inhibit aerobic glycolysis for a longer term treatment (36 hours), the cells begin to “look” unhealthy. Measurements indicate that inhibiting Warburg for these longer periods of time results in LDH accumulation in the media. This enzyme accumulation in the media may be used as a surrogate for cytotoxicity, as LDH is not released from cells unless the membrane has been severely compromised. It is often used as this can indicate general cell death, whether by apoptosis or necrosis and all means of cell death in vitro eventually result in membrane compromise or rupture.

While it appears that there is a loss in cell viability that coincides with PDK inhibition, loss of aerobic glycolysis, and an increase in ROS, our data also demonstrates that the decrease in cell health is indeed ROS mediated. Treating the cells with the

reactive oxygen scavenger (Tempol) and a PDK inhibitor at the same time results in a rescue of cell viability. The Tempol alleviates reactive oxygen stress, scavenging ROS as it is produced in response to inhibition of aerobic glycolysis with Leelamine. This indicates the cell death is most likely due to build-up of ROS and resulting oxidative stress in the cells.

Taken together, our data indicate that inhibition of PDK results in a loss of the Warburg effect, an increase in ROS that results in loss of cell viability in differentiated adipocytes. This indicates a specific role for Warburg effect that is cytoprotective in adipocytes against ROS damage. This also mimics what is observed in tumor cells. As differentiated adipocytes handle large amounts of high energy molecules (fatty acids, glucose, etc) that readily contribute to ROS production when metabolized, it is beneficial for them to have a greater ability to regulate ROS production than their precursors which do not accumulate lipids and demonstrate a much lower flux of glucose.

### **Overall conclusions**

Our data indicate that differentiated human adipocytes indeed produce lactate as part of aerobic glycolysis or the Warburg effect. This metabolic phenotype is not observed in the adipocyte precursors. This phenotype appears to be regulated by PDKs 1 and 4, for which the expression increases in correlation with the observed aerobic glycolysis and inhibition results in loss of the metabolic phenotype. This PDK-regulated Warburg effect is cytoprotective via regulation of ROS production in the differentiated

adipocyte. Thus, taken together, the data support our hypothesis that differentiated adipocytes demonstrate a PDK regulated Warburg-like effect protecting against detrimental elevation of ROS.

### **Impact**

This body of work provides a better metabolic understanding of how and why human adipose tissue lactate production is regulated as observed in clinical studies at a cellular level. While it indicates another role for the PDKs in adipose tissue in addition to its role regulating glyceroneogenesis, it also further bolsters PDKs as a target for cancer therapy as a similar cytoprotective role for aerobic glycolysis is observed in tumor cells. The development of a high content quantitative approach to measure lipid accumulation has generated a better understanding of donor to donor variability in human adipogenic differentiation and the comparative effects of PPAR gamma agonism on modeling adipogenesis from multiple adipose depots. The quantitative method has also been applied to other cell based physiologic and pathophysiologic models.

### **Future work**

Further work elucidating the regulation of PDK expression and thus control of aerobic glycolysis in differentiated adipocytes would be interesting. Data we have generated but not shown in this thesis indicates HIF1 $\alpha$  may indeed be playing a role in this. Protein expression studies focused on nuclear localization would be interesting to determine if HIF1 $\alpha$  accumulation in the nucleus occurs with adipogenic differentiation and correlates with the aerobic glycolytic phenotype and elevated PDK expression.

Chromatin immunoprecipitation studies with HIF1 $\alpha$  would also be interesting to determine whether PDK is indeed under HIF control in the differentiated adipocytes.

As differentiated adipocytes demonstrate a metabolic phenotype somewhat similar to tumor cells, it would be interesting to further investigate other similarities between newly formed adipocytes and tumor cells. We have generated data demonstrating potential differential paracrine regulation of local vasculature between differentiated adipocytes and their precursors that appears somewhat similar to tumor regulation of angiogenesis based on preliminary studies. It would be interesting to look more deeply for similarities between adipocytes in expanding fat mass and cancer cells in the expanding tumor. It would also be particularly interesting to determine if similarities exist between these cells with their ability to switch between oxidative metabolism, glycolytic metabolism, and aerobic glycolysis depending on the hypoxic state of the tumor and the hypoxic or intermittent hypoxic state observed in adipose tissue.

## Chapter IV:

### Experimental Procedures

*ASC isolation and maintenance.* Adipose stem cells (ASCs) were isolated using methods previously described by our lab [254]. Briefly, lipoaspirate (or minced subcutaneous adipose tissue from abdominoplasty) is subjected to collagenase digest and incubated with shaking. Post digest, red blood cell lysis is performed on samples with increased red blood cell content. Adipocytes are then separated from stromal vascular fraction (SVF) by centrifugation. The pelleted SVF is resuspended and passed through a cell strainer to remove excess/undigested tissue. The strained SVF is washed several times and plated on standard tissue culture plastic in EGM2-MV (Lonza) media. Cells are expanded and through subculture when density reaches approximately 75%. For our research, cells underwent two passages and were used for indicated studies at passage three. All metabolic, ROS, and LDH measurements were obtained with cells cultured/treated in DMEM (without glucose/sodium pyruvate/L-glutamine) supplemented with 100 mg/dL glucose unless otherwise noted.

*Adipogenic Differentiation.* For differentiation, ASCs were seeded down into appropriate plates/dishes per experiment at 150000 cells/cm<sup>2</sup> in EGM2-MV(day -2). The following day media was switched to DMEM/F12 containing 10% FBS (day -1).

Following 24 hour incubation, media was switched to DM-2 media from Zen-Bio (day 0) and replaced after three days (day 3). Following an additional three days media was switched to AM-1 media from Zen-Bio (day 6) and changed every three days until use in experiments. Cells obtain an adipocyte phenotype beginning around day 9 and can be used through day 15 though most of our measurements were made on day 12.

*Omental and Subcutaneous ASC matched pair culture and differentiation.*

Omental and subcutaneous ASCs from the same donor were purchased from Zen Bio as a matched pair of ASCs from different depots. Cells were cultured and expanded as described for isolated ASCs. Differentiation was performed as described for isolated ASCs with the addition of PPAR $\gamma$  agonist (from Zen Bio) applied for different durations of differentiation as noted in results.

*Myoblast culture and differentiation into myotubes.* Human primary myoblasts were purchased from Invitrogen and cultured/expanded per vendor recommendation in vendor supplied media. For differentiation or maturation into myotubes, cells were harvested and seeded at high density (150000 cells/cm<sup>2</sup>). The following day, confluent cells were switched to DMEM with 5% horse serum. Media was changed every other day for two weeks for myogenic differentiation.

*Flow cytometry.* Cells were harvested, pellets were resuspended in 4% formaldehyde underwent fixation for 10 minutes at 37°C. Following fixation cells were blocked in 1% BSA. Fixed/blocked cell suspension (1 million cells in 100 ul) was transferred to tubes with appropriate FITC conjugated antibodies and left to incubate at



room temperature in the dark for 1 hour. Cells were pelleted and resuspended in 1 ml PBS and analyzed for fluorescence intensity by flow cytometry.

*Human live adipose tissue imaging.* Subcutaneous human adipose tissue was obtained from abdominoplasty. Tissue was cut into small pieces and stained for 20 minutes in a mixture of isolectin IB4 conjugated to AlexaFluor 488, Nile Red, and Hoechst 33342. After incubation, tissue pieces were washed and immersed in PBS under a coverslip. Imaging was performed using an Olympus FV1000-MPE Confocal/Multiphoton Microscope with dipping objectives to obtain confocal-planar and three-dimensional images of the tissue.

*ROS measurement.* CMH2DCFDA ROS dye was loaded into cells. Briefly, cells were incubated in media containing CMH2DCFDA (10  $\mu$ M) for 20 minutes at 37°C. After loading, the cells were incubated with Nile Red (10nM) and Hoechst 33342 (1:1000) for 20 minutes at 37°C and treated with compounds where appropriate. Live stained cells were then imaged using Leica DMI2000 widefield fluorescence microscope. ROS accumulation was measured as increased CMH2DCFDA fluorescence using a plate based fluorometer at multiple time points post compound addition.

*Lipid accumulation.* Cells were fixed using the Prefer fixative method previously described. Fixed cells were then stained with Nile Red (PBS with 10 nM Nile Red) for 30 minutes at room temperature and then analyzed by laser scanning cytometry (Acumen Explorer) for total lipid fluorescence intensity. Subsequently, samples were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature,

washed, and then incubated with methanol and gentle shaking followed by wash with PBS for several iterations of 20 minutes to reduce lipid in the samples. After lipid extraction and washing, samples were incubated with Hoechst 33342 (1:1000 in PBS with RNase I) for 1 hour at room temperature. Following Hoechst stain, samples were analyzed again by laser scanning cytometry (Acumen Explorer) to obtain total nuclear fluorescence. Total lipid fluorescence was normalized to total nuclear fluorescence for each population. To image lipid accumulation, undifferentiated and differentiated ASCs were fixed by removal of media and addition of Prefer Fixative for 20 minutes at room temperature followed by two PBS washes. After washing, cells were incubated with Nile Red/Syto16 solution (10 nM Nile Red, 1:1000 Syto16 in PBS) for 30 minutes at room temperature. Cells were washed twice and imaged using LEICA DMI2002 widefield fluorescence microscope.

*Imaging myogenic differentiation.* Cells were fixed at various time points throughout differentiation by removal of media and addition of Prefer Fixative for 20 minutes at room temperature followed by two PBS washes. Fixed cells were then permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature, followed by two PBS washes and 1 hour of blocking with 1.0% BSA at room temperature. Primary antibodies were added and incubated at 4°C overnight. The following day, cells were washed twice with PBS followed by incubation with a mixture of appropriate secondary antibodies conjugated to R-phycoerytherin, Phalloidin-Alexa Fluor 488, and Hoechst 33342 for 2 hours at room temperature. Stained cells were washed twice and imaged using LEICA DMI2002 widefield fluorescence microscope. Fluorescence intensity

measurements were obtained laser scanning cytometry as previously described for lipid accumulation measurements.

*Adipokine secretion.* Conditioned media was collected at multiple time points throughout differentiation at 72 hour intervals. Conditioned media was subsequently analyzed by bead based ELISA (Millipore) to determine concentration of adipokines leptin and adiponectin per vendor protocol.

*Lactate measurement.* Both undifferentiated and differentiated cells were incubated in reduced serum (0.1%) DMEM for 4 hours and then incubated in DMEM (without glucose/sodium pyruvate/L-glutamine) supplemented with 100, 500, or 1000 mg/dL glucose for 12 hours with or without the addition of 100 nM insulin. Conditioned media was then collected and analyzed for lactate content using a colorimetric assay (Sigma Aldrich). Conditioned media samples were compared against non-conditioned control media and  $\Delta$  lactate was determined. Values were normalized by time to achieve a lactate production rate per whole population (well).

*Oxygen consumption measurements.* Both undifferentiated and differentiated cells were incubated in reduced serum (0.1%) DMEM for 4 hours and then incubated in DMEM (without glucose/sodium pyruvate/L-glutamine) supplemented with 100, 500, or 1000 mg/dL glucose with or without the addition of 100 nM insulin and subsequently analyzed using Seahorse XF flux analyzer to determine oxygen consumption rates (OCR). For undifferentiated and differentiated myotubes, similar measurements were made but only in DMEM (without glucose/sodium pyruvate/L-glutamine) supplemented with

100 mg/dL glucose. Mitochondrial toxins were injected at various times during OCR measurements as described in results to characterize mitochondrial oxidative capacity.

*Transcript analysis.* Undifferentiated and differentiated adipocytes were harvested for transcript analysis with Trizol. RNA concentrations were measured with Qubit® Fluorometer (Invitrogen). Reverse transcription of 200ng RNA to total cDNA for each sample was carried out using TaqMan® Reverse Transcription Reagents (Applied Biosystems). Quantitative PCR (qPCR) was performed with gene-specific primers (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems), using the Applied Biosystems HT7900 PCR system.

*Western blotting.* Undifferentiated and differentiated adipocytes were harvested for western blotting using 1X RIPA buffer with protease and phosphatase inhibitor cocktails. Lysed samples were flash frozen using liquid nitrogen. Samples were thawed and all subsequent steps were performed on ice. Samples were centrifuged 5 minutes. After centrifugation, lipid layer is discarded (top layer) and samples sit on ice for 20 minutes followed by centrifugation and removal of lipid layer. This is repeated until samples are clear. Protein was determined using BCA assay. 20 ug of protein was separated by electrophoresis in 4-20% Tris glycine gels (in reduced conditions) then transferred to PVDF using iBlot quick transfer (Invitrogen). Membranes were blocked with LiCor blocking buffer for 1 hour. Membranes were then incubated in blocking solution containing primary antibodies overnight at 4°C. Membranes were washed 5 times for 20 minutes in PBST (Tween) and then incubated in blocking buffer with

appropriate secondary LiCor antibodies (IR dye-conjugated). Membranes were again washed 5 times for 20 minutes in PBST and finally in PBS w/o calcium and magnesium. Blots were imaged in LiCor Odyssey. Band intensities were determined using LiCor analysis software and compared against actin bands.

*Cell viability.* Cell viability was measured using fluorescence CytoTox-One LDH release assay (Promega). Differentiated adipocytes were incubated in various treatments as noted in Results. After 36 hour incubation at 37°C, media was collected. Media was then analyzed for LDH accumulation per vendor protocol.

## Chapter V:

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## **Chapter VII:**

### **Curriculum Vitae**

**William Christopher Roell**

#### **Education**

2005-2015

Indiana University, Indianapolis, Indiana

Ph.D. Cellular and Integrative Physiology

Concentration: Diabetes and Obesity

Thesis: PDK Regulated Warburg Effect Protects  
Differentiated Adipocytes Against ROS

1996-2000

Miami University, Oxford, Ohio

B.A. Microbiology, minor in Biochemistry

#### **Work Experience**

2000-2015

Research Scientist, Tailored Therapeutics, Eli Lilly and  
Company

#### **Honors and Awards**

2006

Moenkhaus Award (for exemplary academic record),  
Department of Cellular and Integrative Physiology,  
Indiana University School of Medicine

#### **Grants and Fellowships**

1995

Howard Hughes Fellowship, Miami University, Oxford,  
OH

## Publications

Dage JL, Colvin EM, Fouillet A, Langron E, **Roell WC**, Li J, Mathur SX, Mogg AJ, Schmitt MG, Felder CC, Merchant KM, Isaac J, Broad LM, Sher E, Ursu D. Pharmacological characterisation of ligand- and voltage-gated ion channels expressed in human iPSC-derived forebrain neurons. *Psychopharmacology*. 2014 Mar;231(6):1105-24.

Rajashekhar G, Traktuev DO, **Roell WC**, Johnstone BH, Merfeld-Clauss S, Van Natta B, Rosen ED, March KL, Clauss M. IFATS collection: Adipose stromal cell differentiation is reduced by endothelial cell contact and paracrine communication: role of canonical Wnt signaling. *Stem Cells*. 2008 Oct;26(10):2674-81.

Payne GA, Borbouse L, Bratz IN, **Roell WC**, Bohlen HG, Dick GM, Tune JD. Endogenous adipose-derived factors diminish coronary endothelial function via inhibition of nitric oxide synthase. *Microcirculation*. 2008 Jul;15(5):417-26.

## Abstracts

**Roell WC**, Searfoss G, Baker T, Leishman D, Dage J, Stevens J. Assessing contractility in cardiomyocytes derived from human induced pluripotent stem cells. (Invited talk: Functional cardiovascular assessment in laboratory models. OSU, 2012)

**Roell WC**, Lu Y, Searfoss G, Schmitt M, Baker T, Leishman D, Dage J, Stevens J. Assessing contractility in human IPS cardiomyocytes. (PHARMA/Eli Lilly and Co. Summit, 2012)

**Roell WC**, Kriauciunas A, Schmitt M, Cockerham S, Ruan X, Owens RA, Thomas MK, Dage J. Human adult progenitor cell-derived and iPS cell-derived adipocyte and hepatocyte models for diabetes drug discovery. (PHARMA/Eli Lilly and Co. Summit, 2012)

**Roell WC**, Harris RA, March KL. Aerobic glycolysis reduces oxidative stress in differentiated adipocytes. (3rd Annual Roudebush VAMC Research Symposium, 2011).

**Roell WC**, Kriauciunas A. Non-destructive determination of lipid accumulation using high content analysis. (Invited talk: AUGM, Broad Institute, 2010)

**Roell WC**, Harris RA, March KL. Differentiating adipocytes are protected from ROS via a PDK regulated Warburg Effect. (Indiana Center for Vascular Biology and Medicine Retreat, 2010)

**Roell WC**. Modeling adipogenesis. (Invited talk: Eli Lilly and Co. Stem Cell Summit, 2008)

**Roell WC**, Merfeld S, Johnstone B, McClure D, March KL. Adipogenic Differentiation of ASCs is Associated with Markedly Increased Glucose Consumption and Release of the Vasculotropic Factors VEGF and MCP-1. (IFATS, 2007)

**Roell WC**, Merfeld S, Johnstone B, March KL. Autocrine and paracrine signaling in adipogenesis. (IFATS, 2006)

## Research Experience

Cell Culture: Primary isolation and culture of multiple cell types (human/rodent adipocytes and adipose stromal vascular cells, human/rodent skeletal muscle myoblasts, human/rodent/simian hepatocytes, human/rodent PBMCs, rodent cortical neurons, human/rodent/simian spleenocytes); human Induced Pluripotent Cell (IPS) and differentiated human IPS cell models (parent lines, hepatocytes, cortical neurons, skeletal muscle cells, cardiomyocytes, differentiated intestinal organoid culture); cell lines (HepG2, Hep3b, H42E, SKnMC, CHO, HEK293, L6, C2C12, C3H10T.5, 3T3L1, NHDF, HuVEC, HMVEC)

Preparation and analysis of Protein:  
SDS-PAGE, Western blot analysis, whole-cell immunofluorescence, immunoprecipitation, fluorescent, confocal microscopy, and laser scanning cytometry in cultured cells and intact tissue, subcellular fractionation

Preparation and analysis of DNA and RNA:  
PCR, RT-PCR, qRT-PCR, sequencing, agarose and acrylamide gel electrophoresis, plasmid isolation, restriction digest, ligation, vector construction, isolation of genomic DNA from various tissues

Modeling Human/Rodent Cellular

Differentiation: Hepatic, cardiac, adipocyte, skeletal muscle, neural